Supplying dairy cows with *Propionibacterium* to reduce enteric methane emission

Tilskudd av *Propionibacterium* til melkekyr for å redusere utslipp av enterisk metan

Philosophiae Doctor (PhD) Thesis

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Ås, December 2020

Jikun Chen

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SUMMARY

The primary target of this research was to explore the ability of promising propionibacteria strains to reduce the enteric methane emission from dairy cows. The second objective was to study the effect of propionibacteria on ruminal propionate production and the milk production.

The objective of our first study was to investigate numerous propionic acid bacteria (most of them are propionibacteria) isolates for their ability to affect the production of methane, volatile fatty acids, and substrate degradation *in vitro*. Thirty-one strains were screened for the effects on the methane production and volatile fatty acids concentrations in incubated rumen fluid from non-lactating dairy cows. Two strains inoculated showed capability to mitigate methane emission by up to 20% (v/v). Seven strains could promote ($P < 0.05$) total volatile fatty acids production and five strains stimulated ($P < 0.05$) the yield of propionate. Seven strains promoted ($P < 0.05$) the substrate degradation. *Propionibacterium thoenii* T159 substantially mitigated ruminal methane emission by 20%, and elevated ($P < 0.05$) overall substrate degradation by 8%. Moreover, this strain elevated total ruminal production of volatile fatty acids by 21%, compared with the control group. From these *in vitro* results we concluded that *Propionibacterium thoenii* T159

was the most promising strain to be used in subsequent *in vivo* studies.

The objective of our second study was to determine the ability of *Propionibacterium thoenii* T159 to establish in the rumen of dairy cows at high feeding level. The strain *Propionibacterium thoenii* T159 (5×10^{11} CFU/head × day) was infused into the rumen of four cows via rumen cannula for eight days. After the propionibacteria inoculation ceased, we could still find that three of four donor cows presented substantially increased total population of the propionibacteria (10^6 CFU/mL rumen content) in the rumen as determined by PCR. The present work showed that strain *Propionibacterium thoenii* T159 was able to persist for at least five days in the rumen of dairy cows at high feed intake.

The objective of our third study was to examine the efficacy of the strain *Propionibacterium thoenii* T159 to mitigate enteric methane emissions, to alter rumen fermentation and to improve the performance of dairy cows in a cyclic change-over design. This study consisted of two subsequent experiments with five intact cows (exp1) and four rumen cannulated cows (exp2). The strain *Propionibacterium thoenii* T159 (8.5×10^{11} CFU / head × day) was inoculated into the rumen via esophageal tubing in exp1 and through cannulas in exp2. In both trials the strain *Propionibacterium thoenii* T159 failed to mitigate enteric methane emission and did not increase milk production. Feed intake, rumen fermentation

pattern and digestibility were not, or only negligibly affected by *Propionibacterium thoenii* T159. In conclusion, the methane mitigation potential of *Propionibacterium thoenii* T159 shown *in vitro,* could not be confirmed *in vivo*.

SAMMENDRAG

Hovedmålet med denne forskningen var å utforske om lovende *Propionibacterium* (PB)-stammer kunne redusere utslippet av enterisk metan fra melkekyr. Delmål var å undersøke om PB kunne øke produksjonen av propionsyre i vomma og melkeproduksjonen.

I den første studien var målet å undersøke mange propionsyrebakterie (PAB)-isolater for evnen til å påvirke produksjonen av metan og flyktige fettsyrer (Volatile fatty acids= VFA), samt nedbrytningen/fordøyelsen av substratet *in vitro.* Trettien stammer ble testet for effekten på metanproduksjon og konsentrasjon av VFA i inkubert vomvæske fra ikke-lakterende mjølkekyr. To stammer reduserte metanutslippet med inntil 20% (volum / volum). Syn stammer økte ($P < 0,05$) total VFA-produksjon, og fem stammer økte ($P < 0,05$) produksjonen av propionat. Syn stammer økte ($P < 0,05$) nedbrytingen/fordøyelsen av substratet. *Propionibacterium thoenii* T159 reduserte metanutslippet med hele 20%, og økte ($P <0,05$) nedbrytningen/fordøyelsen av substratet med 8% samt økte den totale VFA-produksjonen med 21%, sammenlignet med kontrollgruppen. Med bakgrunn i disse resultatene oppnådd *in vitro* konkluderte vi med at *Propionibacterium thoenii* T159, som var den mest lovende stammen, skulle brukes i de påfølgende *in vivo*-studiene.

Hovedhensikten med den andre studien var å undersøke om *Propionibacterium thoenii* T159 kunne etablere seg i vomma til melkekyr på høyt fôrnivå. Stammen *Propionibacterium thoenii* T159 (5×10^{11} CFU/ku × dag) ble tilført vomma til fire kyr i 8 dager. Etter at PB-tilførselen stoppet, viste tre av de fire kyrne fortsatt betydelig forhøyet totalt antall PB (10^6 CFU / ml vommen innhold) bestemt ved PCR. Forsøket viste at *Propionibacterium thoenii* T159 var etablert i minst fem dager i vomma til melkekyr på høyt fôrnivå.

I den tredje studien var hensikten å undersøke om *Propionibacterium thoenii* T159 reduserte utslippet av enterisk metan, påvirket gjæringsmønstret i vomma og økte ytelsen til mjølkekyr i et syklisk ombyttingsforsøk. Denne studien besto av to påfølgende eksperimenter med fem intakte kyr (Forsøk 1) og fire vomfistulerte kyr (Forsøk 2). Stammen *Propionibacterium thoenii* T159 ($8{,}5 \times 10^{11}$ CFU/ku × dag) ble tilført vomma via slange i Forsøk 1 og gjennom vomfistel i Forsøk 2. *Propionibacterium thoenii* T159 viste ingen effekt hverken på utslippet av enterisk metan eller mjølkeproduksjonen i de to forsøka. Opptaket av fôr, gjæringsmønster i vomma og fordøyeligheten av rasjonen ble ikke, eller bare ubetydelig påvirket av *Propionibacterium thoenii* T159. Det er konkludert med at den reduksjonen i utslippet av metan oppnådd med T 159 *in vitro* ikke kunne verifiseres *in vivo*.

LIST OF PAPERS

I. **Jikun Chen**, Odd Magne Harstad, Tim McAllister, Peter Dörsch and Helge Holo (2020). Propionic acid bacteria enhance ruminal feed degradation and reduce methane production *in vitro*. Acta Agriculturae Scandinavica, Section A — Animal Science, 69: 3, 169-175.

II. **Jikun Chen**, Helge Holo, Angela Schwarm and Odd Magne Harstad (2020). Ruminal survival of *Propionibacterium thoenii* T159 in dairy cows at high feed intake. Acta Agriculturae Scandinavica, Section A — Animal Science, 69: 3, 189-191.

III. **Jikun Chen**, Angela Schwarm, Tormod Ådnøy, Peter Dörsch, Helge Holo, and Odd Magne Harstad. Effect of *Propionibacterium thoenii* T159 on methane emission, ruminal characteristics, and milk production in dairy cows at high feed intake. (*Manuscript*)

ABBREVIATIONS

A : P	The mole ratio of acetate to propionate
(A + B) : P	The mole ratio of (acetate + butyrate)/propionate
CPI	Crude protein intake
DM	Dry matter
DMI	Dry matter Intake
ECM	Energy-corrected milk
FCM	Fat-corrected milk
F : C	The ratio of forage to concentrate
MG	Methanogens
MGS	Methanogenesis
PB	Propionibacteria
VFA	Volatile fatty acids, also known as SCFA

1. BACKGROUND, OBJECTIVES AND HYPOTHESES

The enteric methane from ruminants accounts for approximately 6% of the anthropogenic greenhouse gas emissions globally (Beauchemin et al., 2020). The methane losses account for 2% to 12% of the gross energy consumed by ruminants (Johnson and Johnson, 1995). Because of the increase in the global population from 2010 to 2050, an elevated global demand for meat and milk is estimated of 73% and 58%, respectively (Beauchemin et al., 2020). Our challenge is how to decrease the energy loss from methane emission of ruminants and increase the animal performance by the administration of probiotics to meet the growing needs of the meat and milk globally.

As one of the less conventional probiotics for domestic animals, propionibacteria is a propionate producer that can utilize hydrogen and pyruvate/lactate to produce propionate anaerobically (Piveteau et al., 1999; Vorobjeva et al., 1999).

The primary target of this study was to investigate the ability of promising propionibacteria strains to mitigate the enteric methane emission of dairy cows. The second objective was to explore the effect of propionibacteria on propionate production in the rumen and the milk production of dairy cattle.

The hypotheses of this study were:

(1) The propionibacteria strains (n = 31) differ in their capability to reduce methane production and to shift the volatile fatty acid pattern in a screening experiment *in vitro*.

(2) The propionibacteria could survive for at least five days in the rumen of dairy cows at high dry matter intake.

(3) The promising propionibacteria strain selected from the *in vitro* experiment reduces enteric methane emission from dairy cows and increase their productive performance.

2. GENERAL INTRODUCTION

The general introduction consists of two main sections. In the first section, the rumen microbiota and the probiotics for animals are introduced in general. As an indigenous inhabitant in the rumen, the biological characteristics of propionibacteria and their possible effects on the rumen fermentation are specified in this section as well. In the second section, the principle of the methanogenesis by methanogens in the rumen and the approach to mitigate the enteric methane emission by microorganisms are illustrated. In the last part, the techniques of methane measurements *in vitro* and *in vivo* are elucidated. The tables in the introduction exhibit an overview of the parameters reported so far and thereby highlight the gaps of knowledge in this field.

2.1 Probiotics

Probiotics are a source of live, naturally occurring microorganisms (Seo et al., 2010) which beneficially affect the hosts by improving their gastrointestinal microbial balance (Yosi et al., 2020). Probiotics are also referred to as direct-fed microbials (DFM) by FDA (1995).

Propionibacteria (*Propionibacterium* spp.) is a kind of probiotics for domestic animals. In the following three sections, the rumen microbiota, the biological characteristics

of propionibacteria, and the studies of the propionibacteria administration for ruminants are elucidated successively.

2.1.1 Rumen microbiota and propionibacteria

The rumen is an essential digestive organ of ruminants, and the main location for dietary fiber (DF) fermentation. Rumen microbiota consists of archaea, bacteria, fungi, protozoa and phages, etc. The rumen harbors more than 200 species of bacteria (McSweeney et al., 2005). Rumen bacteria are mostly Gram-negative, strictly anaerobic with the optimal pH 6.5 ~ 6.9 (Mitsumori and Minato, 1997).

Propionibacteria is a kind of facultative anaerobic actinobacteria which produce propionate as one of the end products in the anaerobic fermentation (Gonzalez-Garcia et al., 2017). They can grow at 15 ~ 40 °C (Vorobjeva, 1999), pH 5.1 ~ 8.5 (Campaniello et al., 2015) and could be divided into two groups (Zarate and Perez, 2015) by their habitats: dairy propionibacteria and cutaneous propionibacteria. Dairy propionibacteria grow optimally at 28 °C to 30 °C and pH 6.5 to 7.0. They are the potential probiotics for animals (Zarate and Perez, 2015).

Propionibacteria is one of the rumen indigenous inhabitants in the population of $10^3 \sim 10^4$ CFU per gram of wet rumen content (Davidson, 1998), which account for 1.4% of total rumen microbes (Azzaz et al., 2019), and 4.3% of rumen

epimural bacteria (Mead and Jones, 1981; Vyas et al., 2014a). Generally, there are two species of propionibacteria which occur in the rumen: *Propionibacterium acidipropionici* (96%) and *P. jensenii* (4%) (Davidson, 1998). Propionibacteria can adhere to rumen epithelium (Zarate et al., 2002).

In the rumen, propionibacteria could produce propionate anaerobically (Figure 2.1). In the first step, pyruvate, methylmalony-CoA and carbon dioxide will be carboxylated to oxaloacetic acid (OAA), accompanied by several mid-products of the citric acid cycle. Afterwards, OAA is reduced to succinate, succinyl-CoA and propionyl-CoA from malate and fumarate. Finally, succinate is converted to propionate and succinyl-CoA by CoA transferase in the Wood-Werkman cycle (Piveteau et al., 1999; Gonzalez-Garcia et al., 2017).

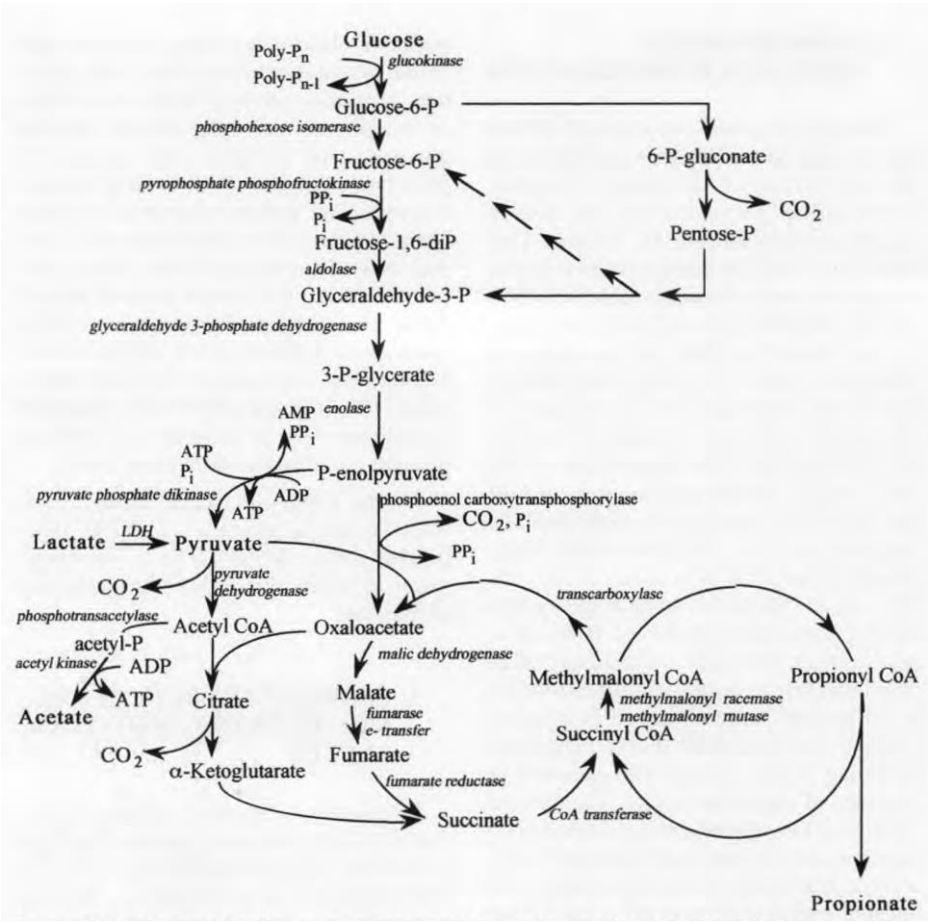

Figure 2.1 Pathways of propionate formation in microbial fermentation. LHD: lactate dehydrogenase; Poly–P_n: polyphosphate; PP_i: pyrophosphate. For reasons of clarity, only the pyrophosphate dependent conversion of fructose–6–P to fructose–1, 6–diP is shown and ATP generation by the electron transfer system is omitted. All the reactions are directed towards propionate production, even though the reactions are reversible (Piveteau et al., 1999).

Propionibacteria produce not only propionate, but also acetate in the lactate fermentation (Zarate and Perez 2015). The metabolic activities of propionibacteria in the rumen can influence ruminal pH. The ruminal pH is mainly determined by the total amount of acetate and propionate, because the level of ruminal lactate is low (Russell et al., 1998). Methanogens cannot absorb H_2 effectively in the rumen when pH is low (Van Kessel et al., 1996), and it could decrease the ruminal ammonia level and mitigating enteric methane emission (Lana et al., 1998). Wood-Werkman cycle (Figure 2.1) is the major and efficient pathway for propionate production by propionibacteria (Gonzalez-Garcia et al., 2017). Theoretically, 1.5 mol of glucose can produce up to 6.0 mol ATP with two possible ATP syntheses by sodium gradients (Figure 2.2) (Pritchard, 1977).

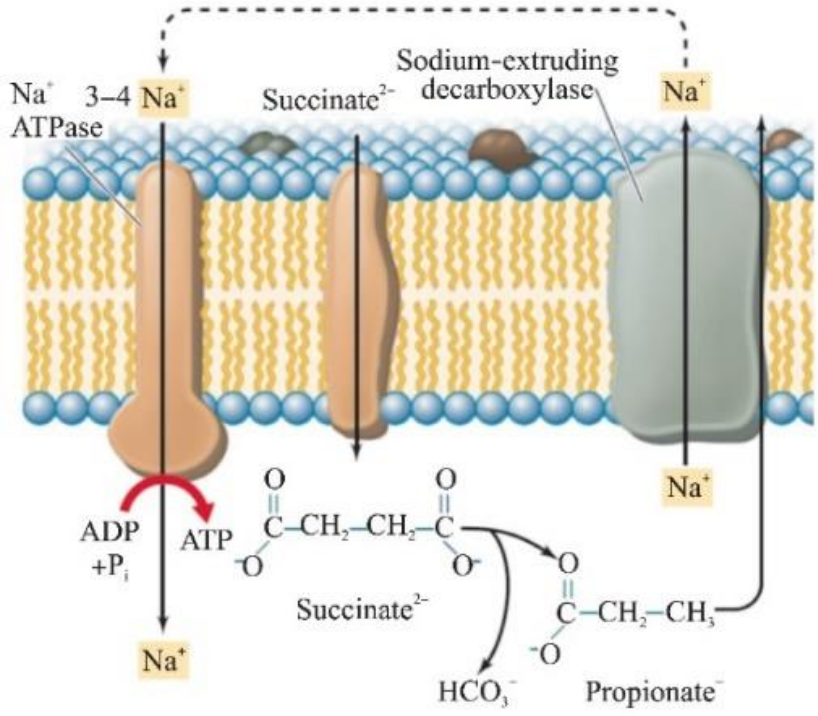

Figure 2.2 The process of microbial propionate formation (Chen et al., 2019).

A high population of propionibacteria in the rumen might be helpful in the improvement of the rumen fermentation pattern and/or mitigate the enteric methane emission. Until now, there are four reports of the propionibacteria administration *in vivo* (Vyas et al., 2014a; Vyas et al., 2014b; Vyas et al., 2016; Jeyanathan et al., 2019) demonstrated the relative abundance, resistance and maintenance of the propionibacteria in the rumen by qPCR.

2.1.2 Effects of propionibacteria on ruminants

As one of the indigenous inhabitants in the rumen, specific strains of propionibacteria could be utilized as a kind of probiotics to increase the relative abundance of propionibacteria and the propionate level in the rumen.

Several researchers have investigated the effect of the propionibacteria on the rumen fermentation, digestibility and performance of ruminants. These previous studies have been performed *in vitro* and *in vivo* and the rumen fermentation parameters are summarized in Table 2.1a, 2.2a (*in vitro*) and Tables 2.1b, 2.2b, 2.3 (*in vivo*). Most of the reports showed the effects of *Propionibacterium freudenreichii*, *P. acidipropionici* and *P. jensenii* strains on the cattle. Only a few studies *in vitro* are available so far and only two studies *in vitro* included a larger screening of different propionibacteria strains *in vitro* (Table 2.1a). However, these two larger screenings used the rumen fluid from beef cattle which are fed differently than dairy cows and therefore a different response could be expected. Hence, the batch culture *in vitro* by using the rumen fluid from dairy cattle is necessary to fill the gaps of the research work recently.

Propionibacteria may increase the propionate production in the rumen and alter ruminal volatile fatty acids profile *in vivo* (Table 2.1b, 2.2b and 2.3). Glucose is one of the major fuel supplies for ruminants and propionate is one of the essential precursors for gluconeogenesis (Drackley et al., 1999),

accounting for 61 ~ 67% of the total glucose synthesis of the cattle (Thompson, 2011). Ruminal propionate could increase blood glucose and reduce DMI, stimulating insulin production and oxidative metabolism, and consequently preserve glycogenic amino acids for protein synthesis, which may lower the maintenance costs of metabolizable proteins and avoid the energy loss (Stein et al., 2006). Propionate could be utilized in the citric acid cycle in the form of acetyl-CoA (Lehloenya et al., 2008b).

According to Table 2.1a and 2.1b, three reports (Yang et al., 2004; Luo et al., 2012; Alazzeh et al., 2013) and two reports (Ghorbani et al., 2002; Vyas et al., 2016) focused on the rumen fermentation parameters of *P. thoenii* strains *in vitro* and *in vivo*, respectively. Two studies focused on one specific *P. thoenii* strain P15 *in vitro* (Yang et al., 2004) and *in vivo* (Ghorbani et al., 2002) in beef cattle, respectively. In Table 2.1b, Vyas et al. (2016) reported the effect of the *Propionibacterium thoenii* T159 on DMI, ruminal pH and ruminal fermentation characteristics *in vivo*. In Table 2.2b and Table 2.3, only a few studies focused on the digestibility and the performance of the cattle by the administration of propionibacteria *in vivo*, however, there is no report on the administration of *Propionibacterium thoenii* strains *in vivo* until recently.

Therefore, it is essential for us to investigate the effect of the administration of *Propionibacterium thoenii* T159 on

dairy cow, especially the effect on the enteric methane emission, milk production, nitrogen balance and digestibility *in vivo*, which was not reported previously.

Table 2.1a Overview of studies on the effect of propionibacteria on ruminal fermentation parameters *in vitro*

Strain	Cattle	pH	VFA	A	P	B	A:P	L	AM	Reference
Propionibacterium freudenreichii P63	Beef	+	-	-	-	-	-	+	-	Parrott, 1997
P. acidipropionici P5	Dairy	-	-	+	+	+	+	-	-	Akay, 2001
P. thoenii P15	Beef	+	+	+	+	+	+	+	+	Yang, 2004
P. freudenreichii P63	Dairy	+	+	+	+	+	+	-	-	Dolecheck, 2011
P. acidipropionici [1]; *P. freudenreichii* [2]; *P. propionicus* [3]; *P. jensenii* [4]; *P. thoenii* [5]	Beef	+	-	+	+	+	+	+	-	Luo, 2012
P. freudenreichii [6]; *P. jensenii* [7]; *P. japonicus* [8]; *P. propionicus* [9]; *P. thoenii* [10]	Beef	-	-	+	+	+	-	-	-	Alazzeh, 2013
P. acidipropionici P169	Heifer	-	+	+	+	+	+	-	-	Alazzeh, 2014
P. freudenreichii NP24	Dairy	+	+	+	+	+	+	-	+	Meale, 2014
P. freudenreichii subsp. shermanii ATCC 8262	Dairy	-	+	+	+	+	+	-	-	Ding, 2017

+: data reported; -: data not reported; Cattle: The type of cattle used in the trial; VFA: Molar concentration of volatile fatty acid (mM); A: Molar proportion of acetate (mol/100 mol volatile fatty acids); P: Molar proportion of propionate (mol/100 mol volatile fatty acids); B: Molar proportion of butyrate(mol/100 mol volatile fatty acids); L: Molar concentration of lactate (mM); AM: Molar concentration of ammonia (mM); *P. acidipropionici* [1]: strain 341 and ATCC 25562; *P. freudenreichii* [2]: strain CSCC 2206 and CSCC 2207; *P. propionicus* [3]: strain T7, T37, T83 and T90; *P. jensenii* [4]: strain 702 and NCFB 572; *P. thoenii* [5]: ATCC 4874; *P. freudenreichii* [6]: strain T5, T28, T31, T54, T114 and T146; *P. jensenii* [7]: strain T1, T121, T122 and T130; *P. japonicus* [8]: strain T7 and T37; *P. propionicus* [9]: strain T83 and T90; *P. thoenii* [10]: strain T159 and ATCC4874.

Table 2.1b Overview of studies on the effect of propionibacteria on ruminal fermentation parameters *in vivo*

Strain	Cattle	pH	VFA	A	P	B	A:P	L	AM	Reference
Propionibacterium freudenreichii P63	Beef	+	-	-	-	-	-	+	-	Parrott, 1997
Propionibacterium spp.[1]	Beef	+	+	+	+	+	+	-	-	Davidson, 1998
P. thoenii P15	Beef	+	+	+	+	+	+	+	+	Ghorbani, 2002
P. acidipropionici DH42	Beef	+	-	+	+	-	+	+	-	Kim, 2002
P. jensenii P169	Dairy	+	-	+	+	+	-	-	-	Stein, 2004
P. jensenii P169	Dairy	+	-	+	+	+	-	-	-	Stein, 2006
P. jensenii P169	Dairy	+	+	+	+	+	+	+	+	Lehloenya, 2008b
P. freudenreichii NP24	Dairy	+	+	+	+	+	+	+	+	Raeth-Knight, 2007
P. jensenii P169	Dairy	-	-	+	+	+	+	-	-	Weiss, 2008
P. jensenii P169	Beef	+	+	+	+	+	+	+	+	Lehloenya, 2008a
P. freudenreichii NP24	Dairy	+	+	+	+	+	+	+	+	Thompson, 2011
P. acidipropionici P169	Beef	+	+	+	+	+	+	+	+	Narvaez, 2014
P. acidipropionici P169	Heifer	+	+	+	+	-	+	-	+	Sanchez, 2014
P. acidipropionici P169; *P. acidipropionici* P5; *P. jensenii* P54	Heifer	+	+	+	+	+	+	-	+	Vyas, 2014a
P. acidipropionici P169; *P. acidipropionici* P5; *P. jensenii* P54	Heifer	+	+	+	+	+	+	+	+	Vyas, 2014b
P. freudenreichii T114; *P. freudenreichii* T54; *P. thoenii* T159	Heifer	+	+	+	+	+	+	+	+	Vyas, 2016
P. freudenreichii NP24	Dairy	+	-	+	+	+	+	-	-	Dickey, 2016
P. acidipropionici P169	Beef	-	+	-	-	-	-	-	+	Azad, 2017
P. freudenreichii P63	Dairy	+	+	+	+	+	+	+	+	Philippeau, 2017
P. freudenreichii 53-W (DSM 20271)	Dairy	+	+	+	+	+	+	-	-	Jeyanathan, 2019

+: data reported; -: data not reported; Cattle: The type of cattle used in the trial; VFA: Molar concentration of volatile fatty acid (mM); A: Molar proportion of acetate (mol/100 mol volatile fatty acids); P: Molar proportion of propionate (mol/100 mol volatile fatty acids); B: Molar proportion of butyrate (mol/100 mol volatile fatty acids); L: Molar concentration of lactate (mM); AM: Molar concentration ammonia (mM); *Propionibacterium* spp.[1]: Unidentified strains P54, P81, P89 and P104.

Table 2.2a Overview of studies on the effect of propionibacteria on substrate degradation *in vitro*

Strain	Cattle	DMD	OMD	ADFD	NDFD	SRD	Reference
Propionibacterium acidipropionici P5	Dairy	+	-	-	+	+	Akay, 2001
P. thoenii P15	Beef	-	-	+	+	+	Yang, 2004
P. freudenreichii NP24	Beef	+	-	-	-	-	Meale, 2014

+: data reported; -: data not reported; Cattle: The type of cattle used in the trial; DMD: Dry matter degradability (%); OMD: Organic matter degradability (%); ADFD: Acid detergent fiber degradability (%); NDFD: Neutral detergent fiber degradability (%); SRD: Starch degradability (%).

Table 2.2b Overview of studies on the effect of propionibacteria on feed digestibility in cattle (*in vivo)*

Strain	Cattle	DMD	OMD	CPD	NDFD	ADFD	SRD	Reference
P. freudenreichii ssp. Shermanii JS (DSM 7067)	Dairy	+	-	-	-	-	-	Jatkauskas, 2006
P. freudenreichii NP24	Dairy	+	-	+	+	-	+	Reath-Knight, 2007
P. jensenii P169	Beef	-	+	-	+	+	-	Lehloenya, 2008a
P. acidipropionici	Beef	+	-	+	+	-	-	Kamarloiy, 2008
P. freudenreichii	Dairy	+	-	+	+	+	-	Arriola, 2011
P. freudenreichii NP24	Dairy	+	-	+	+	+	-	Byod, 2011
P. acidipropionici P169	Heifer	-	-	-	+	+	-	Sanchez, 2014
P. acidipropionici P169; *P. acidipropionici* P5; *P. jensenii* P54	Heifer	+	+	+	+	+	+	Vyas, 2014a
P. acidipropionici P169; *P. acidipropionici* P5; *P. jensenii* P54	Heifer	+	+	+	+	+	+	Vyas, 2014b
P. freudenreichii P169 & Saccharomyces cerevisiae SC	Buffalo	+	+	+	+	+	-	Azzaz, 2015
P. freudenreichii NP24	Dairy	-	+	-	+	-	+	Dickey, 2016
P. freudenreichii P63	Dairy	+	+	-	+	+	-	Philippeau, 2017

+: data reported; -: data not reported; Cattle: The type of cattle used in the trial; DMD: Dry matter digestibility (%); OMD: Organic matter digestibility (%); CPD: Crude protein digestibility (%); NDFD: Neutral detergent fiber digestibility (%); ADFD: Acid detergent fiber digestibility (%); SRD: Starch digestibility (%).

Table 2.3 Overview of studies on the effect of propionibacteria on the performance of dairy cow

Strain	MYD	ECM	MLA	MPT	MF	MUN	ECM/DMI	MYD/DMI	Reference
Propionibacterium jensenii P169	+	+	+	+	+	+	+	+	Francisco, 2002
P. jensenii P169	-	+	+	+	+	+	-	-	Stein, 2006
P. freudenreichii ssp. shermanii JS, DSM 7067	-	+	-	+	+	-	-	+	Jatkauskas, 2006
P. jensenii P169	+	-	+	+	+	+	-	-	Lehloenya, 2008b
P. freudenreichii NP24	+	+	+	+	+	+	-	-	Raeth- Knight, 2007
P. freudenreichii P169	+	-	+	+	+	+	-	-	Ondarza, 2008
P. jensenii P169	+	-	+	+	+	+	-	-	Weiss, 2008
P. freudenreichii NP24	+	-	-	+	+	-	+	+	Boyd, 2009
P. freudenreichii NP24	+	+	-	+	+	-	+	+	Boyd, 2011
P. freudenreichii NP24	+	-	+	+	+	+	-	-	Thompson, 2011
P. frendenreichii	+	-	-	+	+	-	-	-	Vibhute, 2011
P. freudenreichii NP24	+	+	-	+	+	-	+	-	West, 2011
P. freudenreichii NP24	+	-	-	+	+	+	-	-	Ferraretto, 2015
P. freudenreichii, P169	+	-	+	+	+	+	-	-	Sawall, 2015
P. freudenreichii NP24	+	-	-	+	+	+	+	+	Dickey, 2016
P. freudenreichii	+	-	+	+	+	+	-	-	Sawant, 2016
P. freudenreichii P63	+	-	-	+	+	+	-	-	Philippeau, 2017
P. freudenreichii 53-W (DSM 20271)	+	+	-	+	+	-	+	-	Jeyanathan, 2019

+: data reported; -: data not reported; MYD: Milk yield per head per day (kg); ECM: Energy-corrected milk per head per day (kg); MLA: Concentration of milk lactose (%) or milk lactose yield (kg/day); MPT: Concentration of milk protein (%) or milk protein yield (kg/day); MF: Concentration of milk fat (%) or milk fat yield (kg/day); MUN: Concentration of milk urea nitrogen (mM); DMI: Dry matter intake (kg).

2.2 Methane formation and measurement in ruminants

Carbon dioxide, methane, and nitrous oxide are three essential greenhouse gas in the atmosphere which contribute to 77%, 14% and 8% of total global greenhouse gas emission, respectively (IPCC 2019). Despite of the lower atmospheric concentration of methane which is only 0.49% of carbon dioxide, the total global greenhouse effect of all the methane in the atmosphere is 25% of carbon dioxide (Zhao et al., 1999). The residence time of methane in the atmosphere could be approximately 9 to 12 years. Therefore, methane contributes significantly to the global warming.

Enteric methane emissions from ruminants account for 17% of global methane emissions (Knapp et al., 2014), and 80% of total methane emissions from livestock (Gill et al., 2010).

2.2.1 Enteric methane emissions

Generally, approximately 89% of enteric methane from ruminants is released into the atmosphere by eructation (Thorpe, 2009). Methanogens (MG) play an irreplaceable role in the methanogenesis (MGS) of the rumen. Methanogens are a kind of strictly anaerobic archaea in the rumen, which account for less than 1% of total rumen microbes (Wright and Klieve, 2011). The metabolism and the maintenance of the population of methanogens are influenced by the diet, feeding frequency, and the management of the ruminants (Kumar et al., 2011, 2013; Sirohi et al., 2013). All methanogens in the rumen contribute to enteric methane emission (Morgavi et al., 2010).

Due to the low threshold for hydrogen partial pressure (P_{H2}) and fast doubling time, methanogens can grow easily in the optimal growing condition of pH 7.0 and mesophilic temperature in the rumen (Cheng et al., 2013). Most methanogens can utilize hydrogen as the electron donor to reduce carbon dioxide and produce methane in the rumen (Cersosimo and Wright, 2015). Additionally, a few species of methanogens in the rumen could also utilize hydrogen to reduce formate, methanol and methylamine, and produce methane in the rumen (Cheng et al., 2013).

Ruminal hydrogenotrophic methanogens could establish a symbiotic association with hydrogen producers, such as

protozoa, anerobic fungi and hydrogen-producing bacteria in the rumen (Lange et al., 2005).

The methanogenesis and enteric methane emission intimately associate with protozoa positively in the rumen (Beauchemin et al., 2020). The cytoplasm of protozoa contains hydrogenosomes which produce hydrogen as a kind of byproduct in the metabolism of acetate or butyrate (Williams and Coleman 1992). However, when the intracellular hydrogen of the protozoa reaches a high level, there will be a feedback suppression in the metabolism of the protozoa and therefore hydrogen in the cytoplasm of the protozoa needs to be eliminated soon (Zheng and Chen, 2003). Most methanogens can be swallowed by protozoa and remain alive in the cytoplasm as the endosymbiotic methanogens to eliminate the hydrogen for protozoa (Finlay et al., 1994). In the mutually beneficial symbiotic relationship, the endosymbiotic methanogens could utilize hydrogen produced by the intracellular hydrogenosome of the protozoa directly and rapidly (Feng et al., 2004).

In addition, there is a small population of methanogens which live on the cell membrane of protozoa, and are ectosymbiotic methanogens (Feng et al., 2004). Both ectosymbiotic and endosymbiotic methanogens could produce methane by interspecies hydrogen transfer with protozoa (Ng, 2016). Nevertheless, endosymbiotic methanogens are in higher population and they are more

important in the metabolism of ruminal methanogenesis than that of ectosymbiotic methanogens (Feng et al., 2004).

Furthermore, several strains of methanogens in the rumen could acquire electrons from hydrogen by syntrophic metabolism with cellulolytic bacteria or ruminal anaerobic fungi (Kumar et al., 2015), which produce hydrogen in the process of degrading plant cell wall in the diet (Feng et al., 2004).

2.2.2 Mitigate the methane emission by propionibacteria

As a reducing agent, hydrogen is a meaningful restrictive factor in the formation of methane. Besides, enzyme is another factor to limit the rate of the methanogenesis in the rumen (Ungerfeld, 2020). In recent years , there are several microbial additives options for researchers that can help to avoid the incorporation of hydrogen into the methanogenesis. In principle, there are two main approaches to mitigate methane formation (Ungerfeld, 2020):

a) Microbials can incorporate hydrogen into the pathways other than methanogenesis;
b) Microbials can avoid producing hydrogen in the pathway of reduction.

The approach a) is applicable for ruminants in the scientific research and practical use in agriculture, since it might be a challenge to manipulate the metabolism of hydrogen producing microbes in the rumen (Cai et al., 2020). Consequently, by the infusion of the living propionibacteria strain culture into the rumen, propionibacteria might be able to compete for hydrogen with methanogens by reducing fumarate to succinate, thereby diminish methanogenesis. Therefore, the propionate production in the rumen may be promoted, and the energy utilization of ruminants could be improved.

Compared with the methanogenesis, the microbial production of propionate by propionibacteria indicates significant advantages in the thermodynamics of chemical reactions (Table 2.4). The reaction between hydrogen and the electron acceptor is a redox reaction with a redox pair. The kinetics of a redox pair depends on the thermodynamic function of the reaction and Gibbs free energy ($\Delta G_0'$, kJ/mol). The lower ΔG is, the easier the reaction could proceed, and more energy will be released during the chemical reaction. If $\Delta G_0' > 0$, the chemical reaction cannot proceed spontaneously.

Table 2.4 shows the ΔE_0 and $\Delta G_0'$ of the two redox couples. Fumarate is an electron acceptor in the reaction of which redox potential is higher than carbon dioxide. Due to the advantages in the redox reaction thermodynamics, fumarate could obtain the electrons from hydrogen anaerobically, and then produce propionate. In addition, the P_{H2} threshold required in the succinate formation is much lower than that of methanogenesis by the reduction of carbon dioxide (Table 2.4).

Theoretically, propionibacteria may be capable to compete with the methanogens for hydrogen and promote ruminal propionate formation in the rumen. Until now, only three studies reported the effect of propionibacteria on methane production *in vitro* (Table 2.5a), whereof only one includes a larger screening of different propionibacteria strains

performed with rumen fluid from beef cattle. Only six studies reported the effect of propionibacteria on methane production from cattle *in vivo*, only two of which (Table 2.5b) were performed with dairy cows. Until now, the effect of the strain T159 on enteric methane emission of cattle was only investigated in beef cow *in vivo* (Vyas et al., 2016), and the results of milk production and nitrogen balance are absent. Therefore, it is worthwhile for us to explore the effect of *Propionibacterium thoenii* T159 in batch culture *in vitro* and the administration in dairy cow *in vivo*.

Table 2.4 Standard reduction potentials of several common redox couples at pH = 7.0 and Gibbs free energy liberated between hydrogen and two electron acceptors (Prescott et al., 2010; Attwood and McSweeney, 2008; Tratnyek and Macalady, 2000)

Redox couples	**Reduction Half-reaction**	**ΔE_0 (mV)**	**$\Delta G^{0'}$ (kJ/mol)**	**Threshold of H_2 (ppm)**
Carbon dioxide/Methane	$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$	− 238	− 68.3	28.0 ~ 100
Fumarate/Succinate	$HOOCCH{=}CHCOOH + 2H^+ + 2e^- \rightarrow HOOC(CH_2)_2COOH$	+ 32.0	− 86.2	0.90

ΔE_0: Standard reduction-oxidation potential (mV); $\Delta G^{0'}$: Standard free energy change (kJ/mol).

Table 2.5a Overview of studies on the effect of propionibacteria on methane production *in vitro*

Strain	Cattle	Gas	Gas/DM	CH_4	CH_4/DM	CH_4/DMD	CM	Reference
Propionibacterium freudenreichii P63	Dairy	-	-	-	-	-	+	Dolecheck, 2011
P. freudenreichii [1]; *P. jensenii* [2]; *P. japonicus* [3]; *P. propionicus* [4]; *P. thoenii* [5]	Beef	-	+	-	+	-	-	Alazzeh, 2013
P. acidipropionici P169	Heifer	-	+	-	+	-	-	Alazzeh, 2014
P. freudenreichii NP24	Dairy	+	-	+	+	+	-	Meale, 2014
P. freudenreichii subsp. shermanii ATCC 8262	Dairy	+	+	-	+	+	-	Ding, 2017

+: Data reported; -: Data not reported; Cattle: The type of donor animal used in the trial; Gas: The amount of gas per vial per day (mL); CH_4: The methane emission per vial per day (mL); DM: Dry matter of substrate added per vial (g); DMD: Dry matter digested per vial (g); CM: The amount of methane emission per vial per day (mmol); [1]*P. freudenreichii*: strain T5, T28, T31, T54, T114, T146; [2]*P. jensenii*: strain T1, T121, T122, T130; [3]*P. japonicus*: strain T7, T37; [4]*P. propionicus*: strain T83, T90; [5]*P. thoenii*: strain T159, ATCC4874.

Table 2.5b Overview of studies on the effect of propionibacteria on the methane production *in vivo*

Strain	Animal	CH_4	CH_4/ DMI	CH_4/ OMI	CH_4/ MYD	CH_4/ ECM	CH_4/ FCM	CH_4/ GEI	Reference
P. acidipropionici P169; *P. acidipropionici* P5; *P. jensenii* P54	Beef	+	+	-	-	-	-	+	Vyas, 2014a
P. acidipropionici P169; *P. acidipropionici* P5; *P. jensenii* P54	Beef	+	+	-	-	-	-	+	Vyas, 2014b
P. freudenreichii T114; *P. thoenii* T159; *P. freudenreichii* T54	Beef	+	+	-	-	-	-	+	Vyas, 2016
P. freudenreichii 53-W (DSM20271)	Sheep	-	+	-	-	-	-	-	Jeyanathan, 2016
P. freudenreichii P63	Dairy	+	+	+	+	-	+	+	Philippeau, 2017
P. freudenreichii 53-W	Dairy	+	+	-	+	+	-	-	Jeyanathan, 2019

+: Data reported; -: Data not reported; Animal: The type of donor animals used in the trial (beef cow, dairy cow or sheep); CH_4: The methane emission per head per day (g); DMI: Dry matter intake per head per day (kg); OMI: Organic matter intake per head per day (kg); MYD: milk yield per head per day (kg); ECM: Energy-corrected milk per head per day (kg); FCM: 4% Fat-corrected milk per head per day (kg); GEI: General energy intake per head per day (MJ).

2.2.3 Methane measurement techniques

The determination of methane concentration in the gas samples is convenient, accurate and precise by modern instrumental analytical techniques. Therefore, the challenge is the gas sampling from ruminants.

2.2.3.1 Gas sampling technology

General information

In the trials *in vitro*, the rumen fluid from ruminants is transferred to an incubation container with the anaerobic headspace, which is normally an airtight vial sealed by a rubber stopper. After 24 hours or longer time, the gas pressure of the head space is measured, and the gas samples are collected by introducing a gas-tight syringe directly into the vial through the rubber stopper. Then the methane concentration of the gas samples is analyzed by gas chromatography (GC) (Alazzeh et al., 2013; Alazzeh et al., 2014) or other means.

There are several gas sampling techniques *in vivo*. The study which conducted in respiratory calorimetry chambers, such as whole animal chambers, head boxes and face masks, were frequently used before 1990s. The sulphur hexafluoride (SF_6) tracer gas technique was invented in 1990 and became a popular and low-cost mean for researchers to investigate

the enteric methane emission without the need for restraint or enclosure of animals (Beauchemin et al., 2020).

The whole animal chamber is an accurate method for gas sampling. Researchers place one animal in an airtight room and calculate the methane emissions based on the airflow rate and the methane concentration difference between in- and outflowing air of the chamber (Zhao et al., 2011). The advantage of this method is that researchers could measure the enteric methane produced from both rumen and hindgut fermentation which reflects the total enteric emissions of one animal. Therefore, it is the most accurate mean of methane sampling (Zhao et al., 2011). The disadvantage is that the number of animals measured is limited for the availability of airtight rooms, and the cost is high. Furthermore, the methane emissions of ruminants on the pasture cannot be determined by this technique, so the animals measured need to be trained and adaptation for the restraint or enclosure in the chamber in advance (Zhao et al., 2011).

The face mask of the cattle is a convenient method of gas sampling. The mask covers the nose and the mouth of the animals, collecting all the gas around their head. The methane emission is calculated based on the methane concentration of the gas collected and the rate of air flow (Hu et al., 2011). The cost of this method is low, and the operation is simple as well. Therefore, it is suitable for various circumstances in the trial

(Hu et al., 2011). However, the disadvantage is that only the methane exhaled from the mouth and nose of ruminants can be collected, but not the methane emission from hindgut. Furthermore, the breathing mask restrict the eating and drinking behavior of the animals(Hu et al., 2011).

Sulfur hexafluoride tracer method

The sulfur hexafluoride (SF_6) tracer technique is a popular mean of gas sampling from ruminants since 1990s. The advantage is that the researchers could measure the methane emissions for a large quantity of animals at the same time at a low cost. There are some disadvantages. First, it cannot be used in windy locations since the wind can blow away the exhaled methane and lead to inaccurate results. Second, it cannot collect the methane emission from the hindgut of ruminants.

In this study, the sulfur hexafluoride tracer technique was applied (Figure 2.3). According to GB/T 32760-2016 (2016), sulfur hexafluoride can be used as an internal marker in the gas samples. First, researchers measure the permeation rate of the sulfur hexafluoride tube, then place it into the rumen. When animals exhale, the ruminal sulfur hexafluoride tube releases sulfur hexafluoride at a steady low speed. Then the exhaled air from the mouth of donor animals, including sulfur hexafluoride and methane, could be collected into a gas collection device. The device for gas collection is a U-shaped

collection canister like a yoke placed on the neck of animals with the connection of a long metal pipe, the end of which extends to the nose and mouth of animals.

Afterwards, the collected gas samples are analyzed with instruments for the concentration of methane and sulfur hexafluoride. Based on the permeation rate of the sulfur hexafluoride tube, and the concentration of sulfur hexafluoride and methane, the emission rate of methane can be determined. Furthermore, the total amount of methane emission of the animal in a certain period can be calculated.

According to GB/T 32760-2016 (2016), researchers place the sulfur hexafluoride tube into the rumen in the first step. Then researchers should check the air tightness of the gas collection device by flushing which with N_2, and vacuum (−0.1 MPa) it for 3 ~ 4 times. If the gas pressure of the device does not change after 24 hours, it means that there is no gas leakage in the device. Next step, researchers place the gas collection device on the neck of the donor animals and begin to collect the gas samples. After 24 hours, researchers need to replace the gas collection device and collect gas samples in the surroundings as a background control. The gas pressure should be 0.00 ~ 0.05 MPa after collection for 24 hours. Next step, researchers connect the gas collection device to the N_2 bottle and flush N_2 into it until the gas pressure reaches 0.10 ~ 0.15 MPa. Furthermore, researchers collect a certain

amount of gas sample from the device by a syringe and inject it into a pre-prepared vacuum glass vial with a sealed rubber stopper. Finally, the concentration of methane and sulfur hexafluoride in the gas samples should be analyzed within 48 hours.

Hence, the enteric methane production (R_{CH_4}, g/day) is calculated as follows (Lassey, 2013):

$$R_{CH_4} = R_{SF_6} \times \frac{\varphi_{CH_4} - \varphi_{CH_{4b}}}{\varphi_{SF_6} - \varphi_{SF_{6b}}} \times \frac{MW_{CH_4}}{MW_{SF_6}}$$

Where R_{SF_6} is known release rate of sulfur hexafluoride from the permeation tube (g/day); φ_{CH_4} is the methane concentration in the canister (ppm) , φ_{SF_6} is the sulfur hexafluoride concentration in the canister (ppm), $\varphi_{CH_{4b}}$ is the background concentration of methane (ppm), $\varphi_{SF_{6b}}$ is the background concentration of sulfur hexafluoride (ppm); MW_{CH_4} is the molecular mass of methane (16.04 g/mol), and MW_{SF_6} is the molecular mass of sulfur hexafluoride (146.06 g/mol) .

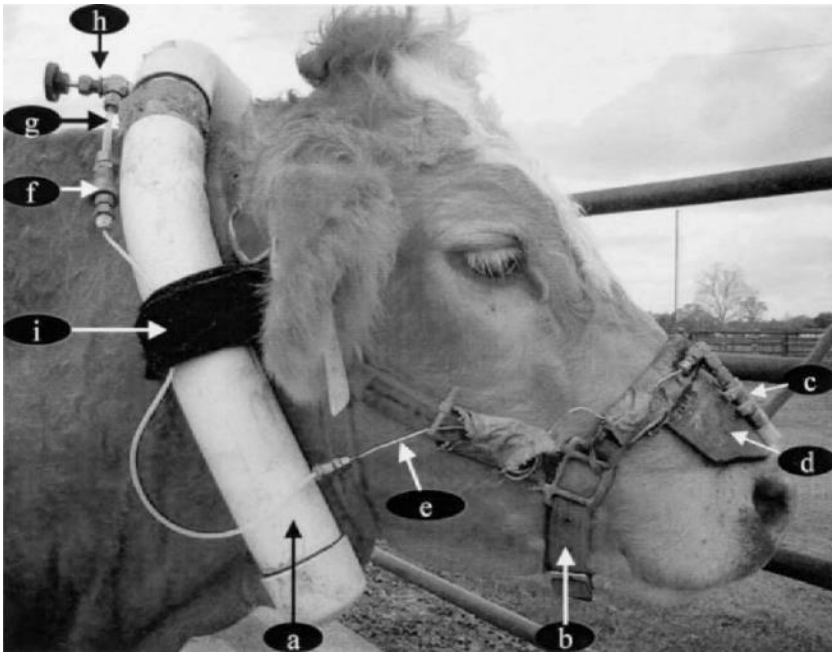

Figure 2.3 Methane collection equipment as worn by a grazing cow: (a) collection canister; (b) halter; (c) filter inlet connected to capillary tubing;(d) leather muzzle protector; (e) stainless steel capillary tubing attached to halter; (f) quick-connect coupling of tubing to canister; (g) Teflon tubing between shut-off valve and quick-connect to capillary tubing; (h) shut-off valve on canister; (i) Velcro strip to anchor canister to halter (DeRamus et al., 2003).

2.2.3.2 The methane determination in the gas samples

The quantification of methane concentration in gas samples is accurate, precise and convenient by automatic analytical instruments.

Gas chromatography (GC) (Liu et al., 2018; Zhou and Wang, 2003), cavity ring-down spectroscopy (CRDS) technique (Chen et al., 2010) and infrared (IR) detectors (Kosterev et al., 1999) can be used for methane quantification in gas samples. Methane gas analyzers (MGA) could be applied for rapid monitoring of methane levels in the wild field and laboratory.

Gas chromatography is a rapid and accurate mean for the methane quantification. The gas samples were separated in the column of gas chromatography according to various capacity of physical adsorption of different gas components. The concentrations of different components in gas samples could be determined by flame ionization detector and thermal conductivity detector separately (Flores et al., 2015). Gas chromatography is used for methane analysis in this study (Chen et al., 2020a; **Paper III**) *in vitro* and *in vivo*.

3. SUMMARY OF PAPERS (I-III)

3.1 Paper I

Propionic acid bacteria enhance ruminal feed degradation and reduce methane production *in vitro*

Methane production represents an energy loss for the ruminants constituting 3 ~ 10% of their gross energy intake. As a kind of probiotics, propionibacteria may have the potential to mitigate the enteric methane emission and improve the milk production of dairy cattle. In this study, using rumen fluid from Norwegian Red dairy cows, we studied many propionic acid bacteria isolates for their ability to affect the production of methane and of the volatile fatty acids, and the ruminal feed degradation *in vitro*.

Thirty-one strains of propionic acid bacteria (most of them are propionibacteria) were screened for their effects on methane production and volatile fatty acid concentrations using *in vitro* assays of rumen fluid from Norwegian dairy cows and a grass silage–concentrate mixture as substrate. Nine strains were further analyzed for effects on substrate degradation.

Propionic acid bacteria led to reductions of up to 20% in methane production. Seven strains stimulated volatile fatty acid production, and in their presence *in vitro* substrate degradation tended to increase ($P < 0.10$). Among all the strains screened in the trial, *Propionibacterium thoenii* T159

could reduce methane production by 20%. In addition, the strain T159 could increase the overall substrate degradation by 8% ($P < 0.05$) and total volatile fatty acid production by 21% ($P < 0.05$).

In conclusion, the strain T159 may be a promising strain to mitigate the enteric methane emission and improve the productive performance of dairy cows.

3.2 Paper II

Ruminal survival of *Propionibacterium thoenii* T159 in dairy cows at high feed intake

Propionibacteria could produce propionate anaerobically and have been applied as probiotics for ruminants to improve their performance. Propionibacteria is a natural inhabitant in the rumen and several previous reports have demonstrated the survival status of the specific inoculated propionibacteria strains in rumen within 24 h.

In this study, *Propionibacterium thoenii* T159 (5×10^{11} CFU/head × day) were administered daily into the rumens of four Norwegian Red cows. Total population of the propionibacteria in the rumen of three cows were substantially increased during and after the treatment with *Propionibacterium thoenii* T159 relative to the background.

In conclusion, the strain *Propionibacterium thoenii* T159 was able to persist for at least five days in the rumen of dairy cows at high dry matter intake (3.9% of body weight).

3.3 Paper III

Effect of *Propionibacterium thoenii* T159 on methane emission, ruminal characteristics, and milk production in dairy cows at high feed intake

In our previous studies, we have found the strain *Propionibacterium thoenii* T159 performed the significant improvement in the ruminal propionate production and methane mitigation *in vitro*. Additionally, we reported that the strain *Propionibacterium thoenii* T159 was able to persist for at least five days in the rumen *in vivo*. Hence, it is worthwhile to examine the effect of strain *Propionibacterium thoenii* T159 administered into the rumen of dairy cow *in vivo*.

The objective of this study was to test the efficacy of the strain *Propionibacterium thoenii* T159 to mitigate enteric methane emissions, to improve the performance of Norwegian Red cows, and to identify interactions between treatment and pretreatment in a changeover design. Two consecutive experiments were conducted with five intact cows and four ruminally cannulated cows fed the same basal diet consisting of 60 : 40 of silage : concentrate (DM basis). The strain *Propionibacterium thoenii* T159 (8.5×10^{11} CFU/head × day) was administrated for the cows to examine the effect of methane mitigation, milk production, milk composition and digestibility. The results showed that *Propionibacterium thoenii* T159 failed to improve rumen

fermentation pattern and milk yield, and to mitigate enteric methane emission.

4. GENERAL DISCUSSION

There are four sections in this discussion. First, the effect of propionibacteria on the rumen fermentation parameters in the trials *in vitro* and *in vivo* is explained. Second, the effect of propionibacteria on dry matter intake, the ruminal methane production, methane emission intensity and their relationship *in vitro* and *in vivo* is illustrated. Third, the effect of propionibacteria on the digestibility *in vitro* and *in vivo* is elucidated. Fourth, the performance of the Norwegian Red cow *in vivo* is discussed. The four sections contain tables summarized the results of previous literature and this study.

4.1 Effects of propionibacteria on volatile fatty acids

In this study *in vitro*, thirty-one strains of propionate producing bacteria were investigated in batch culture (Chen et al., 2020a). In the degradation trials (forage : concentrate ratio of substrate = 60 : 40), three strains (T159, LMGT2832 and LMGT2841) stimulated the molar proportion of propionate ($P < 0.05$) in the total volatile fatty acids *in vitro* (Chen et al., 2020a). Five propionibacteria strains (LMGT2842, LMGT2864, T83, T88 and T93) could stimulate the production of total volatile fatty acids ($P < 0.05$).

Until now, there were twelve studies reported the effect of the propionibacteria administration on the profiles of volatile fatty acids in the rumen (Table 4.1a) *in vitro*. Among these cases, eight reports showed an elevated molar proportion of propionate ($P < 0.05$) *in vitro*. Meantime, three cases showed an elevation of molar proportion of acetate and/or butyrate ($P < 0.05$), indicating that the propionibacteria might stimulate rumen bacteria to produce several intermediates that facilitate the conversion of pyruvate to acetate (Ungerfeld et al., 2005) *in vitro*. On the other side, Sanchez et al. (2014) demonstrated a decrease of molar proportion of acetate by *P. acidipropionici* P169 ($P < 0.05$) with two different substrates (forage : concentrate ratio= 100 : 0 or 50 : 50) *in vitro*. Conversely, Alazzeh et al. (2013, 2014) reported

a significant decline in molar proportion of propionate ($P < 0.05$) within 24 hours in batch culture *in vitro* with two different substrates (forage : concentrate ratio = 100 : 0 or 0 : 100), but the molar proportion of butyrate increased in the trial with the substrate of 100% forage. Meale et al. (2014) and Sanchez et al. (2014) presented an elevation in the molar concentration of total volatile fatty acids ($P < 0.05$) *in vitro*. Furthermore, eight studies *in vitro* reported a declined acetate : propionate ratio by propionibacteria administration (Table 4.1a). By contrast, the previous studies *in vivo* exhibited fewer positive results regarding the improvement in the profiles of volatile fatty acids (Table 4.1b).

Table 4.1a Summary of reported results of the effect of propionibacteria on the rumen fermentation *in vitro*

Reference	Strain	Dose	Diet	Cattle	pH	VFA	A	P	B	A:P	L	AM
Parrott, 1997	*Propionibacterium freudenreichii* P63	1×10^{7}	NR	Beef	+	-	-	-	-	-	+	-
Davidson, 1998	*P. acidipropionici*; *P. freudenreichii*; *P. jensenii*; *P. thoenii*	1×10^{7}	NR	Beef	Hc	-	-	-	-	-	-	-
Akay, 2001	*P. acidipropionici* P5	1×10^{3}, 1×10^{6}, 1×10^{9}	100:0; 0:100	Beef	-	-	Hc	Hc	Hc	Lc	-	-
Yang, 2004	*P. thoenii* P15	2×10^{5}	60:40; 40:60	Beef	+	+	+	+	+	+	+	+
Dolecheck, 2011	*P. freudenreichii* P63	1×10^{6}	60:40; 40:60	Dairy	+	Hb	+	+	Hb	+	-	-
Luo, 2012	*P. acidipropionici* [a]; *P. freudenreichii* [b]; *P. propionicus* [c]; *P. jensenii* [d]; *P. thoenii* [e]	3.3×10^{2}; 3.3×10^{7}	NR	Beef	Ha	-	Ha	Ha	Ha	Lb	La	-
Alazzeh, 2013	*P. freudenreichii* [f]; *P. jensenii* [g]; *P. japonicus* [h]; *P. propionicus* [i]; *P. thoenii* [j]	1.4×10^{8} ~ 6.7×10^{9}	0:100; 100:0	Beef	-	Hc	Lc/Hc	Hc	Lc/Hc	Lc	-	-
					-	Hc	Lc	Hc	Lc/Hc	Lc	-	-
Alazzeh, 2014	*P. acidipropionici* P169	1.2×10^{4}	0:100	Heifer	-	+	+	+	+	+	-	-
		1.2×10^{6}				+	+	+	+	+		
		1.2×10^{8}				+	+	+	+	+		
		1.2×10^{4}	100:0		-	+	+	+	+	+	-	-
		1.2×10^{6}				+	+	+	+	+		
		1.2×10^{8}				+	+	+	+	+		
		2.4×10^{6}	60:40		-	+	+	+	+	+	-	-
		2.4×10^{8}				Hc	+	Hc	+	Lc		
Meale, 2014	*P. freudenreichii* T54	6×10^{9}	15:85	Beef	+	Hb	Lc	Ha	+	La	-	+
Sanchez, 2014	*P. acidipropionici* P169	6×10^{10}	100:0; 50:50	Heifer	-	Ha	La	Ha	-	La		
					-	Ha	+	Ha	-	La		
Ding, 2017	*P. freudenreichii* subsp. shermanii ATCC 8262	2.5×10^{7}	87:13	Dairy	-	+	+	+	+	+	-	-
Chen, 2020a This study	*P. thoenii* T159	2×10^{8} ~ 4×10^{8}	60:40	Dairy	-	Hc	Hc	Hc	Hc	Hc	-	-

+: data reported, insignificant difference with control group; -: data not reported; H: Higher than control; L: Lower than control; a: P < 0.001; b: P < 0.01; c: P < 0.05; Dose: Colony-forming unit of the microbes offered per vial per day; Diet: The ratio of forage to concentrate; Cattle: The type of cattle used in the trial; VFA: Molar concentration of volatile fatty acid (mM); A: Molar proportion of acetate (mol/100 mol volatile fatty acids); P: Molar proportion of propionate (mol/100 mol volatile fatty acids); B: Molar proportion of butyrate(mol/100 mol volatile fatty acids); L: Molar concentration of lactate (mM); AM: Molar concentration of ammonia (mM); NR: No record; *P. acidipropionici* [a]: strain 341, ATCC 25562; *P. freudenreichii* [b]: strain CSCC 2206, CSCC 2207; *P. propionicus* [c]: strain T7, T37, T83, T90; *P. jensenii* [d]: strain 702, NCFB 572; *P. thoenii* [e]:ATCC 4874; *P. freudenreichii* [f]: strain T5, T28, T31, T54, T114, T146; *P. jensenii* [g]: strain T1, T121, T122, T130; *P. japonicus* [h]: strain T7, T37; *P. propionicus* [i]: strain T83, T90; *P. thoenii* [j]: strain T159, ATCC4874.

Table 4.1b Summary of reported results of the effect of propionibacteria on the rumen fermentation *in vivo*

Reference	Strain	Dose	Diet	AT	pH	VFA	A	P	B	A:P	L	AM
Davidson, 1998	*Propionibacterium* spp. [a]	1×10^{7}	10:90; 25:75; 40:60; 55:45; 70:30.	Beef	+	+	+	+	+	+	-	-
Ghorbani, 2002	*P. thoenii* P15	1×10^{10}	9:91	Beef	+	+	+	+	Hc	+	ND	+
Kim, 2002	*P. acidipropionici* DH42	1×10^{7}; 1×10^{8}; 1×10^{9}	9:91	Steer	+	-	Lc	Ha	-	La	+	-
Stein, 2006	*P. jensenii* P169	6×10^{10}; 6×10^{11}	40:60	Dairy	Lc	-	+	Hc	+	Ld	-	-
Raeth-Knight, 2007	*Lactobacillus acidophilus* LA747 & *P. freudenreichii* NP24; *L. acidophilus* LA45 & NP24	NP24: 2×10^{9}; LA747: 1×10^{9}; LA45: 5×10^{9}	60:40; 40:60	Dairy	+	+	+	+	+	+	+	+
Lehloenya, 2008b	*P. jensenii* P169	6×10^{11}	46:54	Dairy	+	+	+	Hc	+	Ld	ND	+
Weiss, 2008	*P. jensenii* P169	6×10^{11}	54:46	Dairy	-	-	Lb	Hc	Hc	Hc	-	-
Thompson, 2011	*P. freudenreichii* NP24	1×10^{9}	36:64	Dairy	+	+	+	+	+	+	+	Lc
Lettat, 2012	*P. freudenreichii* P63	1×10^{11}	80:20	Sheep	La	Lb	Lb	+	Lb	-	Lb	Lb
Ebeid, 2013	*P. freudenreichii* P169	6×10^{9};1.2×10^{10}	30:70	Sheep	+	+	+	+	+	Lc	-	+
Narvaez, 2014	*P. acidipropionici* P169	1×10^{11}	12:88	Steer	+	+	Lc	+	Hb	+	+	Ha
Raphélis-soissan, 2014	*P. acidipropionici*	1.15×10^{11}	NR	Sheep	Hc	Lc	+	+	+	+	-	-
Sanchez, 2014	*P. acidipropionici* P169	6×10^{10}	64:36	Heifer	+	+	+	Ha	-	Hb	-	Ha
Dickey, 2016	Bovamine® *L. acidophilus* NP51 & *P. freudenreichii* NP24	NP51: 1×10^{9}; NP24: 2×10^{9}	36:64	Dairy	+	-	+	+	+	+	-	-
Philippeau, 2017	*P. freudenreichii* P63	1×10^{10}	55:45	Dairy	Hb	+	+	+	+	+	Lc	+
Vyas, 2014a	*P. acidipropionici* P169; *P. acidipropionici* P5; *P. jensenii* P54	5×10^{9}	70:30	Heifer	+	+	+	+	+	+	-	+

Vyas, 2014b	*P. acidipropionici* P169; *P. acidipropionici* P5; *P. jensenii* P54	5×10^{9}	10:90	Heifer	+	+	+	+	+	+	+	+
Vyas, 2016	*P. freudenreichii* T114; *P. freudenreichii* T54; *P. thoenii* T159	1×10^{11}	60:40	Heifer	+	+	Lc (T159)	+	+	Hc (T114)	+	+
Azad, 2017	*P. acidipropionici* P169	1×10^{11}	NR (high corn)	Beef	-	Hc	-	-	-	-	-	Hc
Jeyanathan, 2019	*P. freudenreichii* 53-W (DSM 20271)	1×10^{11}	55:45	Dairy	+	+	+	+	+	+	-	-
Paper III This study	*P. thoenii* T159	8.5×10^{11}	60:40	Dairy	+	+	+	+	Ha	+	+	+

+: data reported, insignificant difference with control group; -: data not reported; H: Higher than control; L: Lower than control; a: $P < 0.001$; b: $P < 0.01$; c: $P < 0.05$; d: $0.05 \leq P < 0.10$; ND: Not detected; Dose: Colony-forming unit of the microbes offered per head per day; Diet: The ratio of forage to concentrate; AT: The type of donor animal used in the trial (beef cattle, dairy cattle, steer, heifer or sheep); NR: No record; VFA: Molar concentration of volatile fatty acid (mM); A: Molar proportion of acetate (mol/100 mol volatile fatty acids); P: Molar proportion of propionate (mol/100 mol volatile fatty acids); B: Molar proportion of butyrate (mol/100 mol volatile fatty acids); L: Molar concentration of lactate (mM); AM: Molar concentration of ammonia (mM); *Propionibacterium* spp. [a]: Unspecified propionibacteria strains: P54, P81, P89, and P104.

In total, twenty-one studies *in vivo*, including this study (**Paper III**), reported the effect of propionibacteria on profiles of volatile fatty acids in livestock, five studies of which observed an elevation ($P < 0.05$) in the molar proportion of propionate *in vivo* by the administration of propionibacteria (Table 4.1b). On the other side, five studies reported a decline ($P < 0.05$) in the molar proportion of acetate by the administration of propionibacteria *in vivo*. Especially, Vyas et al. (2016) demonstrated a declined acetate level ($P < 0.05$) by the strain T159, the identical strain with this study (**Paper III**), in the Canadian ruminally cannulated beef cattle. However, we observed an elevation of acetate level in Norwegian Red cow (67.4 mol/100 mol volatile fatty acids in control group vs. 72.1 mol/100 mol volatile fatty acids in the *Propionibacterium thoenii* T159 group, $P = 0.20$) by the administration of *Propionibacterium thoenii* T159, though we used the diet with the same forage : concentrate (60 : 40) ratio as Vyas et al. (2016). Moreover, four studies, including this study (**Paper III**), showed an elevation of butyrate level ($P < 0.05$) by the administration of propionibacteria, and Lettat et al. (2012) showed a decline of butyrate level ($P < 0.05$) in sheep by the administration of propionibacteria. Azad et al. (2017) showed a significant elevation in the concentration of total volatile fatty acids by the propionibacteria administration. Besides, three studies and four studies reported an elevation and decline of A : P ratio ($P < 0.05$) by

the propionibacteria administration, respectively. Four studies demonstrated a significant difference in ruminal pH by the propionibacteria administration.

Among the five studies *in vivo* showing elevated ruminal propionate level ($P < 0.05$) in the propionibacteria treatment group (Table 4.1b), the feed types were inconsistent, and the proportion of forage in the feed varied from 9% (Kim et al., 2000, Kim et al., 2002) to 53.5% (Weiss et al., 2008), indicating that the different forage : concentrate (F : C) ratio of the diets may have little impact on the metabolic activity of the inoculated propionibacteria in the rumen. Mateos et al. (2015), on the other hand, discovered that for both trials *in vivo* and *in vitro*, the effect of forage on ruminal fermentation could be consistent, and the reason need to be further investigated. Normally, high concentrate diet leads to a high yield of propionate. However, it is not always the case. Patel et al. (2011) only observed the changing of butyrate level *in vivo* accompanied by the different feed types offered to the cattle. Besides, Moss et al. (1995) reported that the propionate level was declined, and the butyrate level was increased with the elevating proportion of concentrate in the diets. Murphy et al. (2000) showed there was no relationship between the proportion of concentrate in the feed and the production of ruminal propionate *in vivo*. Generally, Huhtanen et al. (1998) argued that the degree of silage fermentation for the preparation of the grass silage diets

might affect the volatile fatty acids profiles in the rumen. In summary, there is no consistent evidence that the high-concentrate diet could promote the growth and/or metabolism of the propionibacteria on the propionate production, regardless of the exogenous or indigenous propionibacteria in the rumen. This viewpoint is contrary to Sanchez et al. (2014), who speculated that the high forage diets contained more starch may produce more glucose, and the effect of the propionibacteria on the molar proportion of propionate in the total volatile fatty acids was insignificant subsequently. Nevertheless, although the dairy cows were fed high concentrate (91%) diets (Kim, 2002), propionibacteria may still play a critical role in the elevation of the ruminal propionate level in a short period. However, Reath-Knight et al. (2007) demonstrated an insignificant improvement in total volatile fatty acids and volatile fatty acids profiles by the strain NP24 administration in the dairy cows fed the diet with the same F : C ratio (60 : 40) as this study (**Paper III**).

In Table 4.1b, five trials, including this study (**Paper III**), demonstrated a significant elevation of butyrate level in the propionibacteria group, but few reports revealed a significant lower butyrate level except Lettat et al. (2012) in sheep. Reasonably, the concentration of the total volatile fatty acids is also a slightly higher in T159 treatment group (P = 0.14) than in control group in this study *in vivo* (**Paper III**). Butyrate is absorbed and then significantly inhibit utilization of

propionate by liver, inhibiting gluconeogenesis subsequently (Bergman et al., 1990). In this study (**Paper III**), the molar proportion of propionate in the rumen are higher but not significant in T159 treatment group ($P > 0.10$), it is possible that a large amount of propionate absorbed by rumen epithelium had been transferred to the liver afterwards (Feng et al., 2004). Hence, it might be critical to lower the butyrate level in the rumen while increasing the yield of ruminal propionate. Only in this way could we ensure that most propionate could be utilized in the gluconeogenesis, improving energy status in early lactating period, and offering energy for milk production and metabolisms of cattle afterwards. Ruminal propionate level, however, did not present a significant increase in most of the studies *in vivo*, including this study (**Paper III**). The reason might be related to the metabolic inactivity of the exogenous propionibacteria strains in the propionate fermentation of the rumen.

4.2 Effects of propionibacteria on methane production

According to Table 4.2a, Alazzeh et al. (2013) reported that eight propionibacteria strains, including *Propionibacterium thoenii* T159, could significantly mitigate methane emissions within 24 hours *in vitro*. In this study *in vitro* (Chen, 2020a), three propionibacteria strains (T159, LMGT 2826 and LMGT 2827) demonstrated a decline ($P < 0.05$) in methane yield of 18%, 8% and 20% in batch culture *in vitro*, respectively, compared with the control groups. However, the strain *Propionibacterium thoenii* T159 had no impact on methane yield *in vivo* (**Paper III**), although they may survive and persist in the rumen for five days at least (Chen et al., 2020b). One of the possible reasons is that the strain *Propionibacterium thoenii* T159 may stimulate the metabolism of several indigenous propionate producers in the rumen fluid *in vitro* to promote the propionate production (Alazzeh et al., 2013; Chen et al., 2020b), nevertheless, they might not be able to achieve it *in vivo*.

Table 4.2b summarizes the results of this study (**Paper III**) and previous studies *in vivo*. In this study *in vivo* (**Paper III**), propionibacteria did not significantly mitigate methane emissions in Norwegian Red cows. This is consistent with the previous study by Vyas et al. (2016) who found that *Propionibacterium thoenii* T159 had no impact on daily

methane emissions and methane emission intensity (g/DMI) in Canadian beef cattle fed the mixed diet. and the F : C ratio (60 : 40) of the diet is identical to this study (**Paper III**). Moreover, Vyas et al. (2016) showed similar methane emission intensity (20.0 ~ 24.1 g/DMI) as this study (21.7 ~ 23.3 g/DMI**, Paper III**). However, the average DMI of the Canadian beef cattle (Vyas et al., 2016) is much lower than this study (**Paper III**). One of the possible reasons is that the average methane emission per cow (381 ~ 440 g/day) in this study is much higher than that of Vyas et al. (2016) (172.3 ~ 210.0 g/ day).

Table 4.2a Summary of reported results of the effect of propionibacteria on methane production *in vitro*

Reference	Strain	Dose	Diet	Cattle	Gas/DM	Gas	CH_4	CH_4/DM	CH_4/DMD	CM
Dolecheck, 2011	*P. freudenreichii* P63	1×10^6	60:40; 40:60	Dairy	-	-	-	-	-	+
Alazzeh, 2013	*P. freudenreichii* [a]; *P. jensenii* [b]; *P. japonicus* [c]; *P. propionicus* [d]; *P. thoenii* [e]	1.4×10^8 ~ 6.7×10^9	0:100	Beef	Lc	-	-	Lc	-	-
			100:0		Lc	-	-	Lc	-	-
Alazzeh, 2014	*P. acidipropionici* P169	1.2×10^4	0:100	Heifer	Hc	-	-	Hc	-	-
		1.2×10^6			Hc	-	-	Hc	-	-
		1.2×10^8			Hc	-	-	Hc	-	-
		1.2×10^4	100:0		Hc	-	-	+	-	-
		1.2×10^6			+	-	-	+	-	-
		1.2×10^8			+	-	-	+	-	-
		2.4×10^6	60:40		+	-	-	+	-	-
		2.4×10^8			+	-	-	+	-	-
Meale, 2014	*P. freudenreichii* T54	6×10^9	15:85	Beef	+	+	La	La	La	-
Ding, 2017	*P. freudenreichii* subsp. shermanii ATCC 8262	2.5×10^7	87:13	Dairy	+	+	-	-	-	-
Chen, 2020a This study	*P. thoenii* T159	2×10^8 ~ 4×10^8	60:40	Dairy	+	+	La	La	La	La

+: data reported, insignificant difference with control group; -: data not reported; H: Higher than control; L: Lower than control; a: $P < 0.001$; b: $P < 0.01$; c: $P < 0.05$; d: $0.05 \leq P < 0.10$;; Dose: Colony-forming unit of the microbes offered per vial per day; Diet: The ratio of forage to concentrate; Cattle: The type of cattle used in the trial; Gas: The gas amount per vial per day (mL); CH_4: Daily methane emission per vial per day (g); DM: Dry matter of substrate added per vial (g); DMD: Average dry matter degraded per vial (g); CM: Methane emission per vial per day (mmol); *P. freudenreichii* [a]: strain T5, T28, T31, T54, T114, T146; *P. jensenii* [b]: strain T1, T121, T122, T130; *P. japonicus* [c]: strain T7, T37; *P. propionicus* [d]: strain T83, T90; *P. thoenii* [e]: strain T159, ATCC4874.

Table 4.2b Summary of reported results of the effect of propionibacteria on methane production *in vivo*

Reference	Strain	Dose	Diet	AT	DMI	CH_4	CH_4/ DMI	CH_4/ OMI	CH_4/ MYD	CH_4/ ECM	CH_4/ FCM	CH_4/ GEI
Vyas, 2014a	*P. acidipropionici* P169; *P. acidipropionici* P5; *P. jensenii* P54	5×10^9	70:30	Beef	+	+	Lc	-	-	-	-	+
Vyas, 2014b	*P. acidipropionici* P169; *P. acidipropionici* P5; *P. jensenii* P54	5×10^9	10:90	Beef	+	+	+	-	-	-	-	+
Jeyanathan, 2016	*P. freudenreichii* 53-W (DSM20271); *L. pentosus* D31; *L. bulgaricus* D1	53-W: 6×10^{10}; D31: 6×10^{10}; D1: 3×10^{10}	70:30	Sheep	-	-	+	-	-	-	-	-
Vyas, 2016	*P. freudenreichii* T114; *P. freudenreichii* T54; *P. thoenii* T159	1×10^{11}	60:40	Beef	+	+	+	-	-	-	-	+
Philippeau, 2017	*P. freudenreichii* P63	1×10^{10}	55:45	Dairy	+	+	+	+	+	-	+	+
Jeyanathan, 2019	*P. freudenreichii* 53-W (DSM 20271)	1×10^{11}	55:45	Dairy	+	+	+	-	Hc (high starch)	+	-	-
Paper III This study	*P. thoenii* T159	8.5×10^{11}	60:40	Dairy	+	+	+	-	+	+	-	-

+: data reported, insignificant difference from control group; -: data not reported; H: Higher than control; L: Lower than control; c: $P < 0.05$; d: $0.05 \leq P < 0.10$; Dose: Colony-forming unit of the microbes offered per head per day; Diet: The ratio of forage to concentrate; AT: The type of animal (beef cow, dairy cow or sheep) used in the trial; CH_4: The daily methane emission per head per day (g); DMI: Dry matter intake per head per day (kg); OMI: Organic matter intake per head per day (kg); MYD: milk yield per head per day (kg); ECM: Energy corrected milk per head per day (kg); FCM: 4% Fat-corrected milk per head per day (kg)GEI: General energy intake per head per day (MJ).

Jeyanathan et al. (2019) observed a significant elevation in methane emission intensity (g /kg milk yield) in 55% forage-fed (high starch, 27.4% of the DM basis) dairy cows ($P < 0.05$). Meanwhile, Vyas et al. (2014a,b) fed the beef cattle with different types of diets (F : C = 70 : 30 or 10 : 90) added one of the propionibacteria strains P169, P5 or P54, however, three strains slightly promoted the enteric methane emissions ($P > 0.10$) in Canadian beef cattle. However, due to a higher DMI ($P > 0.10$) in the propionibacteria treatment group (Table 4.2b), there was a slightly decrease ($P > 0.10$) in methane yield (g/DMI) except the strain P54 in beef cattle fed with a corn grain finishing diet (Vyas et al., 2014b). This study *in vivo* (**Paper III**) showed the similar results with a different mixed diet (F : C ratio = 60 : 40). Both methane production and methane yield (g/DMI) showed an increase ($P > 0.10$) in exp1 and a decrease ($P > 0.10$) in exp2 (**Paper III**). Philippeau et al. (2017) reported the cows fed the propionibacteria strain P63 presented no effect (Table 4.2b) in daily methane emission (g/day), methane yield (g/DMI, g/OMI) and methane emission intensity (g/kg milk yield). Nevertheless, Philippeau et al. (2017) reported that methane emission intensity (g/kg 4% fat-corrected milk) by the combined administration of the strains P63 and *Lactobacillus rhamnosus* 32 showed a trend of significant lower result ($0.05 < P \leqq 0.10$) compared with the control groups. The F : C ratio (55 : 45) of the diets in the study of Philippeau et al. (2017) was similar to this study

(**Paper III**) (60 : 40), but the strain (*Propionibacterium freudenreichii* P63 and *Lactobacillus rhamnosus* 32) offered for the dairy cattle was different (Table 4.2b) from this study (**Paper III**). In this study (**Paper III**), we observed the similar methane emission intensity (g/kg milk yield and g/kg ECM) between the T159 treatment group and the control group in exp1. However, in exp2 (**Paper III**) we observed the slightly lower ($P > 0.10$) methane emission intensity (g/kg milk yield and g/kg ECM) in the T159 treatment group than in the control group. These results indicated that *Propionibacterium freudenreichii* P63 may present similar metabolic activity in the propionate production as the strain *Propionibacterium thoenii* T159 (**Paper III**). In summary, all studies *in vivo* (Table 4.2b), including this study (**Paper III**), indicated that the propionibacteria administration for the cattle may not mitigate the enteric methane emissions *in vivo* significantly in a short-period administration, regardless of the cattle type, the diet type, the propionibacteria strain and the daily dose of the strain.

Lehloenya et al. (2008a) reported that propionibacteria strains did not affect the passage rate of ruminal feed particle, rate of rumen fluid and turnaround time. The average DMI and the methane emission *in vivo* were not affected by propionibacteria in most cases (Table 4.2b). Until now, there were six studies (Table 4.2b), including this study (**Paper III**) reporting daily methane yield (g/DMI) of the cattle and only

one study (Vyas et al., 2014a) reported a decline ($P < 0.05$) (25.7 g/DMI in control group, 22.7 g/DMI in P169 treatment group, 23.5 g/DMI in strain P5 treatment group and 22.4 g/DMI in strain P54 treatment group) in the ruminally cannulated cows fed high forage diets (F : C ratio = 70 : 30) among all of the propionibacteria treatment groups. The average DMI (9.2 ~ 9.7 kg/head × day) by Vyas et al. (2014a) was much lower than this study (**Paper II and III**). By contrast, in this study (**Paper III**), the DMI was 17.6 kg/head × day for intact cows and 18.6 ~ 18.8 kg/head × day for ruminally cannulated cows. The methane yield in this study (**Paper III**) was 21.7 ~ 22.0 g/DMI in the intact cows and 22.5 ~ 23.3 g/DMI in the ruminally cannulated cows, a little lower than that of Vyas et al. (2014a). The methane emission (381 ~ 440 g/day) in this study (**Paper III**) was much higher than Vyas et al. (2014a) (167 ~ 190 g/day). This can be explained by the higher feed intake, which is the major driver of methane emission. Feed intake and thus methane emission was numerically higher in cannulated cows than intact cows, which can be explained by the sampling of rumen content via rumen cannula, the lactation number and lactation stage of the cows in the experiments. The F : C ratio of the diets in this study (**Paper I and III**) was 60 : 40, and the forage proportion is lower (Table 4.2b) than Vyas et al. (2014a). Nevertheless, the methane yield (g/DMI) in this study (**Paper III**) was higher than Vyas et al. (2014b) and Vyas et al. (2016),

supporting the higher relevance of feed intake than dietary ingredients. Therefore, we expected that the strain T159 may mitigate the enteric methane emission and increase the milk production in the cattle at a high feed intake level. However, according to the results in this study (**Paper III**), it seems that the effect of propionibacteria on methane emission is not related to the feed intake level of the animals. Vyas et al. (2014b) showed the methane yields between 13.9 ~ 17.9 g/DMI and Vyas et al. (2016) showed the methane yields between 20.0 ~ 22.7 g/DMI in ruminally cannulated beef cattle. Vyas et al. (2016) showed similar methane yield (g/DMI) as this study (**Paper III**). Vyas et al. (2014b) used a diet with a F : C ration of 10 : 90 and Vyas et al. (2016) used a diet with an identical F : C ratio (60 : 40) to this study (**Paper III**). The higher daily methane production (g/day) in this study (**Paper III**) than Vyas et al. (2014a,b, 2016) could be attributed to a higher DMI of Norwegian Red cow than Canadian beef cow. Vyas et al. (2016) showed propionibacteria had no treatment effect on methane yield (g/DMI) in Canadian beef cow. Consistently, strain T159 in this study had no treatment effect on the methane yield (g/DMI) of dairy cows either (**Paper III**). It appears that *Propionibacterium thoenii* T159 may have little impact on the rumen fermentation pattern and the mitigation of methane regardless of the feed intake level.

Moreover, Philippeau et al. (2017) and Jeyanathan et al. (2019) presented a lower average daily methane emission (g/head × day) than in this study *in vivo* (**Paper III**). Both studies showed that the cattle in propionibacteria treatment group had no impact on daily methane emission ($P > 0.10$) (207 g/day in both groups, Philippeau et al., 2017) or increased daily methane emission slightly (291 g/day in control group vs. 310 g/day in strain 53W treatment group, from Jeyanathan et al., 2019). Both studies used the diets of F : C ratio (55 : 45) which was similar to this study (F : C = 60 : 40) (**Paper III**). However, methane yield (g/DMI) in these two trials were quite different, which may be attributed to the different DMI in different breeds of cattle. Philippeau et al. (2017) showed a higher DMI (18.5 kg/day vs. 19.5 kg/day) in propionibacteria treatment group. By contrast, Jeyanathan et al. (2019) showed a lower DMI (12.2 kg/day vs. 12.5 kg/day) in propionibacteria treatment group. The DMI in the study of Philippeau et al. (2017) was similar to this study (**Paper III**), but the DMI in the study of Jeyanathan et al. (2019) was much lower than the DMI in this study (**Paper III**). Hence, the methane yield (g/DMI) in the study of Philippeau et al. (2017) was much lower than Jeyanathan et al. (2019) and this study (**Paper III**). The methane emission intensity (g/kg milk and g/kg ECM) in the study of Philippeau et al. (2017) was also lower than Jeyanathan et al. (2019) and this study (**Paper III**). In addition, the breed of cattle was also different in these

reports mentioned above. Philippeau et al. (2017) and Jeyanathan et al. (2019) used Holstein dairy cows, by contrast, we used Norwegian Red cow in this study (**Paper III**). Different breeds of cattle are featured with diverse methanogens microbiota which might affect the physiological states of ruminal methanogenesis and the enteric methane emission.

As a kind of hydrogenotrophic anaerobic actinobacteria, propionibacteria need to compete for electrons from hydrogen with methanogens in the rumen in order to produce propionate. However, the powerful redox potential of methanogens and their intimate physical relationship with hydrogen donors such as protozoa (< 10 μm), whether by attaching to the cell membrane or by living in the cytoplasm in a symbiotic relationship, could enable methanogens to obtain hydrogen efficiently and rapidly (Hegarty and Gerdes, 1999).

Perhaps this theory could explain the high level of ruminal propionate by long-term (32 weeks) administration of *Propionibacterium jensenii* P169 in the study of Stein et al. (2006, with no methane emission measurements), and the failure of propionate increasing and concomitant mitigating methane emission of this study (**Paper III**) and Vyas et al. (2014a,b, 2016) with the administration of propionibacteria for only a short period (28 days in Vyas 2014a,b; 21 days in Vyas et al., 2016 and this study, **Paper III**). Long-term

administration of exogenous propionibacteria may establish and maintain a high population of living propionibacteria in the rumen microbiota continuously that may affect the fermentation pattern in the rumen significantly in a long period. However, when the administration is only over a short period, the effect on the rumen fermentation and the mitigation of methane may not be established sufficiently.

In this study (Chen et al., 2020b), *Propionibacterium thoenii* T159 survived or stimulated the growth/reproduction of the indigenous propionibacteria in the rumen of dairy cattle for at least five days. Furthermore, we also observed a pretreatment effect for methane emission intensity (g/kg ECM) (**Paper III**), which indicated that the strain T159 may be able to survive in the rumen even much longer than five days in this study *in vivo*. Nevertheless, this might have not been long enough to establish and maintain a high population of the strain in the rumen micro-community sufficiently. The long-term survival of T159 and the long-term effect of T159 on enteric methane emission could be investigated and elucidated further in the future.

Moreover, *Propionibacterium jensenii* LMGT2826 and *Propionibacterium thoenii* LMGT2827 reduced methane yield (g/DMI) *in vitro*, although they did not promote the propionate production. These two strains might be further investigated *in vivo* in the future.

4.3 Effects of propionibacteria on feed degradation

The previous studies of the feed degradation by the administration of propionibacteria *in vitro* and *in vivo* are summarized in Table 4.3a and Table 4.3b, respectively. In this study *in vitro* (Chen et al., 2020a), seven propionibacteria strains increased the degradation of the substrates in batch culture, but the effects were not consistent in total four substrate degradation trials of this study *in vitro*. Two strains (*P. jensenii* LMGT2824 and *P. thoenii* T159) presented a significant elevation in the substrate degradation in two of four trials in this study (Chen et al., 2020a) *in vitro*. By contrast, the strain *Propionibacterium thoenii* T159 did not improve the digestibility of organic matter and neutral detergent fiber *in vivo* (**Paper III**). The inconsistent results of the digestibility between the trials *in vitro* (Chen et al., 2020a) and *in vivo* (**Paper III**) indicated that the strain T159 may not have the ability to improve the rumen fermentation pattern *in vivo*.

Table 4.3a Summary of reported results of the effect of propionibacteria on the substrate degradation *in vitro*

Reference	Strains	Dose	Diet	Cattle	DMD	OMD	ADFD	NDFD	SRD
Akay, 2001	*Propionibacterium acidipropionici* P5	1×10^3, 1×10^6, 1×10^9	100:0; 0:100	Dairy	Lb	-	-	Lb	Hc/Lc
Yang, 2004	*P. thoenii* P15	2×10^5	60:40; 40:60	Beef	+	+	+	+	+
Meale, 2014	*P. freudenreichii* NP24	6×10^9	15:85	Beef	+	-	-	-	-
Chen, 2020a	*P. thoenii* T159	4×10^8	60:40	Dairy	Hc	-	-	-	-
This study									

+: reported, insignificant difference with control group; -: not reported; H: Increased compared to control; L: Decreased compared to control; b: $P < 0.01$; c: $P < 0.05$; Dose: Colony-forming unit of the microbes offered per vial per day; Diet: The ratio of forage to concentrate; Cattle: The type of donor cattle used in the trial; DMD: Dry matter degradability (%); OMD: Organic matter degradability (%); ADFD: Acid detergent fiber degradability (%); NDFD: Neutral detergent fiber degradability (%); SRD: Starch degradability (%).

Table 4.3b Summary of reported results of the effect of propionibacteria on the feed digestibility *in vivo*

Reference	Strain	Dose	Diet	Cattle	DMD	CPD	OMD	ADFD	NDFD	SRD	NI
Jatkauskas, 2006	*P. freudenreichii* ssp. Shermanii JS DSM 7067 & *L. rhamnosus* LC 705 DSM7061	4×10^{10}	75:25	Dairy	+	-	-	-	-	-	-
Reath-Knight, 2007	*Lactobacillus acidophilus* LA747 & *P. freudenreichii* NP24; *L. acidophilus* LA45 & NP24	NP24: 2×10^{9}; LA747: 10^{9}; LA45: 5×10^{9}	60:40; 40:60	Dairy	+	+	-	-	+	+	-
Lehloenya, 2008a	*P. jensenii* P169	6×10^{11}	46:54	Steer	-	-	+	+	+	-	+
Kamarloiy, 2008	*L. plantarum* & *P. acidipropionici*	Unspecified	94.5:5.5	Beef	Hc	Hc	-	Hc	Hc	-	-
Arriola, 2011	*Pediococcus pentosaceus* PP, *P. freudenreichii* PF & *L. buchneri* LB	PP: 1.6×10^{9}; PF: 1.6×10^{9}; LB: 1.6×10^{9}	50:50	Dairy	+	+	-	Lc	Ld	-	-
Byod, 2011	Bovamine® *L. acidophilus* NP51 & *P. freudenreichii* NP24	4×10^{9}, a blend CFU of NP51 and NP24	48:52	Dairy	Hb	Hc	-	Hb	Ha	-	-
Thompson, 2011	Bovamine® *L. acidophilus* NP51 & *P. freudenreichii* NP24	NP51: 1×10^{9} NP24: 1×10^{9}	35.8:64.2	Dairy	+	+	+	+	+	-	-
Morsy, 2014	*P. freudenreichii* P169	6×10^{10}; 6×10^{11}	30:70	Buffalo	+	+	+	+	+	-	-
Sanchez, 2014	*P. acidipropionici* P169	6×10^{10}	64:36	Heifer	-	-	+	-	+	-	-
Vyas, 2014a	*P. acidipropionici* P169; *P. acidipropionici* P5; *P. jensenii* P54	5×10^{9}	70:30	Beef	+	+	+	+	+	-	-
Vyas, 2014b	*P. acidipropionici* P169; *P. acidipropionici* P5; *P. jensenii* P54	5×10^{9}	10:90	Beef	+	+	+	+	+	+	-
Azzaz, 2015	*P. freudenreichii* P169 & *Saccharomyces cerevisiae* SC	P169: 1.2×10^{11}; SC: 1.3×10^{6}	50:50	Buffalo	Hc	Hc	Hc	Hc	Hc	-	-
Dickey, 2016	*P. freudenreichii* NP24	NP51: 1×10^{9}; NP24: 2×10^{9}	36:64	Dairy	-	-	+	-	+	+	+

Philippeau, 2017	*P. freudenreichii* P63	1×10^{10}	55:45	Dairy	+	-	+	+	+	-	-
Paper III This study	*P. thoenii* T159	8.5×10^{11}	60:40	Dairy	-	-	+	-	+	-	+

+: data reported, insignificant difference with control group; -: data not reported; H: Higher than control; L: Lower than control; a: $P < 0.001$; b: $P < 0.01$; c: $P < 0.05$; d: $0.05 \leq P < 0.10$; Dose: Colony-forming unit of the microbes offered per head per day); Diet: The ratio of forage to concentrate; Cattle: The type of donor cattle used in the trial; NR: No record; DMD: Dry matter digestibility (%); CPD: Crude protein digestibility (%); OMD: Organic matter digestibility (%); ADFD: Acid detergent fiber digestibility (%); NDFD: Neutral detergent fiber digestibility (%); SRD: Starch digestibility (%); NI: Data about nitrogen metabolism.

Until now, there are four reports depicting results on the substrate degradation by the administration of propionibacteria *in vitro* (Table 4.3a). Akay et al. (2001) reported a higher starch degradation ($P < 0.01$), and a lower degradation of neutral detergent fiber ($P < 0.01$)and true dry matter ($P < 0.01$) in the strain P5 treatment group (10^9 CFU/g) *in vitro*. Yang et al. (2004) and Meale et al. (2014), however, did not report any significant difference between the control group and the propionibacteria group *in vitro*. These three findings *in vitro* were inconsistent with this study (Chen et al., 2020a) which may be attributed to the different strains of propionibacteria, different doses of the strains, or different rumen fluid used in the trials. Feed digestibility *in vivo* was reported in Table 4.3b. Several studies reported an improved digestibility of several nutrients of feed in cattle. Arriola et al. (2011) reported a lower digestibility of neutral detergent fiber and acid detergent fiber in the propionibacteria group than the control group in the trial *in vivo*. Furthermore, three studies in Table 4.3b showed higher feed digestibility in the trial *in vivo* by the combined administration of several different probiotics (e.g. *Lactobacillus* spp. and *Propionibacterium* spp., *Bifidobacterium* spp. and *Propionibacterium* spp.). The effects of these combinations of several species of probiotics on the digestibility of livestock were unclear and needed to be explored further in the future.

In this study (**Paper III**) *in vivo*, the DMI of cannulated cow in T159 treatment group tended to decrease ($P < 0.10$). Consistently, Ferraretto et al. (2015) showed a trend for a decline of DMI ($P < 0.08$) *in vivo*. Furthermore, Francisco et al. (2002) reported a lower ($P < 0.01$) DMI (g/kg BW) in the dairy cattle fed *P. jensenii* P169 *in vivo*. However, several trials *in vivo* reporting increased digestibility presented an elevation ($P < 0.05$) of DMI (Azzaz et al., 2015; Kamarloiy et al., 2008) by the propionibacteria administration. Several trials reporting a decline of digestibility also exhibited an elevation in the DMI of cattle (Arriola et al., 2011) or a decline in the DMI of buffalo (Morsy et al., 2014) by the administration of propionibacteria. However, we observed a slight decline (approx. 1%) of DMI in the T159 treatment group (exp1: $P < 0.10$; exp2: $P > 0.10$) of this study (**Paper III**). In summary, the strain T159 had no impact on the digestibility of organic matter and neutral detergent fiber in this study *in vivo*. The influence of propionibacteria on the metabolism of rumen microbiota seem to be limited and it might be due to their incapability to compete, integrate in the rumen microbiota and maintain high metabolic activity in the rumen micro-community.

Nevertheless, among all the strains screened in this study *in vitro* (Chen et al., 2020a), *Propionibacterium jensenii* LMGT2824 significantly increased the substrate degradation in two of the four trials *in vitro*. Although *Propionibacterium*

jensenii LMGT2824 did not stimulate the propionate production, it is valuable to investigate the substrate degradation of the strain LMGT2824 in rusitec *in vitro* and in the animal experiment *in vivo* in the future.

4.4 Effects of propionibacteria on animal performance

We hypothesized that the administration of propionibacteria may increase the milk production of dairy cows since propionibacteria may increase the propionate production in the rumen which may promote milk synthesis. The propionate is the primary source of glucose in the ruminants to produce milk (Stein et al., 2006). According to our knowledge, none of the previous reports (Table 4.4) studied the effect of the strain T159 on the milk yield and milk composition of dairy cattle until now. Therefore, this study (**Paper III**) should be first report in this area. Unfortunately, the strain T159 did not presented any significant difference in the milk yield of ruminally cannulated cow in T159 treatment group, while the percentage of lactose in milk and milk urea nitrogen in the ruminally cannulated cows increased significantly in T159 treatment group ($P < 0.05$), which suggested that the propionibacteria strain may manipulate the milk lactose level and affect the way of absorbing serum milk urea nitrogen by mammary gland (Stein et al., 2006). However, we did not observe the similar effect in the intact cows of exp1 (**Paper III**).

The previous studies (Table 4.4) which reported the effect of the administration of propionibacteria on the performance of dairy cows are summarized in Table 4.4, including this

study (**Paper III**). Among these cases, several studies reported a significant increase of milk yield (kg/day) by the administration of the propionibacteria strains. However, Weiss et al., (2008) reported that *Propionibacterium jensenii* P169 (6×10^{11} CFU/head × day) caused milk depression of Holstein dairy cow fed a mixed diet (F : C = 60 : 40) in a 17-week administration. By contrast, Stein et al. (2006) reported that *P. jensenii* P169 (6×10^{10} and 6×10^{11} CFU/head × day) could increase ($P < 0.003$) the milk yield (4% fat-corrected milk, kg/day) of Holstein dairy cow fed a mixed diet (F : C = 53.5 : 46.5) in a 32-week administration. The difference could be attributed to the different diets between the study of Stein et al. (2006) and Weiss et al. (2008). However, in this study *in vivo*, the strain T159 had little impact on the milk production except the percentage of milk lactose and the molar concentration of urea content in milk which are differed between exp1 and exp2 in this study (**Paper III**). Compared with the milk composition of the intact cows in exp1, the significantly higher milk lactose and urea content ($P < 0.05$) in T159 supplied cows in exp2 might be attributed to the rumen content sampling of the ruminally cannulated cows. However, the reason needs to be explored further in the future.

Table 4.4 Summary of reported results of the effect of propionibacteria on the performance of dairy cows

Reference	Strain	Dose	Diet	DMI	MYD	ECM	MLA	MPT	MF	MUN	ED	MD
Francisco, 2002	*Propionibacterium jensenii* P169	6×10^{10}	53:47	Lb	+	+	+	Hb	+	+	+	+
Stein, 2006	*P. jensenii* P169	6×10^{10}; 6×10^{11}	40:60	-	-	-	Hb	Ha	Hc	Hc	-	-
Jatkauskas, 2006	*P. freudenreichii* ssp. Shermanii JS (DSM 7067)	4×10^{10}	75:25	+	+	+	-	+	+	-	-	-
Lehloenya, 2008b	*P. jensenii* P169	6×10^{11}	46:54	-	Hc	-	Hd	Hc (true protein)	Lc	+	-	-
Raeth-Knight, 2007	*P. freudenreichii* NP24	NP24: 2×10^{9}; LA747: 1×10^{9}; LA45: 5×10^{9}	60:40; 40:60	+	+	+	+	+	+	+	+	-
Ondarza, 2008	*P. freudenreichii* P169	6×10^{10}	NR	Hc	Hc	-	+	Hb (true protein)	+	+	-	-
Weiss, 2008	*P. jensenii* P169	6×10^{11}	53.5:46.5	Lc	+	+	+	+	+	+	+	-
Boyd, 2011	Bovamine® *L. acidophilus* NP51 & *P. freudenreichii* NP24	4×10^{9}, a blend CFU of NP51 and NP24	48:52	+	Hb	Hc	-	Hd (true protein)	+	-	+	+
Thompson, 2011	*P. freudenreichii* NP24	1×10^{9}	35.8:64.2	+	+	-	+	+	+	+	-	-
Vibhute, 2011	*P. frendenreichii* PF, *L. acidophilus* LA, *S. cerevisiae* SC-47 & *S. boulardii* SB	PF: 5×10^{8}, 7.5×10^{8}, 1×10^{9}; SC-47: 3×10^{9}, 4.5×10^{9}, 6×10^{9}; SB: 5×10^{8}, 7.5×10^{8}, 1×10^{9}; LA: 4.5×10^{8}, 6.75×10^{8}, 9×10^{8}	40:60	-	+	-	-	+	+	-	-	-
West, 2011	*L. acidophilus* NP45, *P. freudenreichii* NP24 & *L. Acidophilus* NP51	NP24: 2×10^{9}; NP51: 5×10^{8}, 1×10^{9}; NP45: 5×10^{8}	40:60	+	Hd	Hc	-	+	Hc (true fat)	-	Hc	-
Morsy, 2014 (Buffalo)	*P. Freudenreichii,* Pro P169	6×10^{10}; 6×10^{11}	30:70	Lc	+	-	+	+	+	-	-	-
Azzaz, 2015 (Buffalo)	*P. Freudenreichii,* Pro P169 & *Saccharomyces cerevisiae* SC	P169: 1.2×10^{11}; SC: 1.3×10^{6}	50:50	Hc	Hc	-	Hc	Hc	Hc	-	-	-

Ferraretto, 2015	Bovamine® *L. acidophilus* NP51 & *P. freudenreichii* NP24	NP51: 1×10^{9}; NP24: 2×10^{9}	54:46	Ld	+	-	-	+	+	+	-	-
Sawall, 2015	*P. freudenreichii,* P169	6×10^{11}	78:13	-	Hc (high starch)	+	+	+	+	+	+	-
Dickey, 2016	Bovamine® *L. acidophilus* NP51 & *P. freudenreichii* NP24	NP51: 10^{9}; NP24: 2×10^{9}	36:64	+	Hc	Hb	-	Ha (true protein)	Hc (true fat)	Ha	+	Hd
Sawant, 2016	Biovet® *L. acidophilus* LA, *S. cerevisiae* SC, *S. boulardii* SB & *Propionibacterium freudenreichii* PF	LA: 2.25×10^{10}; SC: 1.5×10^{11}; SB: 2.5×10^{10}; PF: 2.5×10^{10}	97:3	-	+	-	-	+	+	-	-	-
Philippeau, 2017	*P. freudenreichii* P63	1×10^{10}	55:45	+	+	-	-	+	+	+	-	-
Jeyanathan, 2019	*P. freudenreichii* 53-W (DSM 20271)	1×10^{11}	55:45	+	+	+	-	+	+	-	+	-
Paper III This study	*P. thoenii* T159	8.5×10^{11}	60:40	+	+	+	+	+	+	+	+	-

+: data reported, insignificant difference with control group; -: data not reported; H: Higher than control; L: Lower than control; a: $P < 0.001$; b: $P < 0.01$; c: $P < 0.05$; d: $0.05 \leq P < 0.10$; Dose: Colony-forming unit of the microbes offered per head per Day; Diet: The ratio of forage to concentrate; NR: No record; DMI: Dry matter intake per head per day (kg); MYD: Milk yield per head per day (kg); ECM: Energy-corrected milk per head per day (kg); MLA: Concentration of milk lactose (%) or milk lactose yield (kg/day); MPT: Concentration of milk protein (%) or milk protein yield (kg/day); MF: Concentration of milk fat (%) or milk fat yield (kg/day); MUN: Concentration of milk urea nitrogen (mM); ED: Energy-corrected milk (kg) per kg of dry matter intake; MD: Milk yield (kg) per kg of dry matter intake; True protein: The total nitrogen of milk minus the nonprotein nitrogen (kg/day); True fat: The fat content of milk (kg/day).

All the previous reports with positive or no effect on the milk production offered a similar dose of the propionibacteria strains (mostly $10^9 \sim 10^{11}$ CFU/head × day) to the cattle in the trials, which was consistent to this study (**Paper III**) (8.5 × 10^{11}/head × day). There was no difference in the forage to concentrate proportion of the diets between the cases with positive effect and the cases with no effect on the milk production. In this study (**Paper III**), the cows were fed with high forage (F : C = 60 : 40) diets. In the previous cases with positive effect, the F : C of the diets were between 40 : 60 and 97 : 3, and those cases which showed no effect on the milk production by the administration of the propionibacteria strains *in vivo* also used the diet with different F : C ratios. The only difference between the cases with positive effects and the cases with no effect is the administration period. However, although we discovered that the long-term trials (32 weeks) may demonstrate high possibility of positive effect *in vivo* (Stein et al., 2006; Lehloenya et al., 2008b), there were a few cases in short period that showed positive effects (Table 4.4), for instance, Azzaz et al. (2015) and Sawant et al. (2016) presented the elevation of the milk production by the administration of the propionibacteria strains *in vivo* for 8 weeks and 6 weeks, respectively. However, the duration of these trials was still much longer than this study (**Paper III**) (3 weeks). By contrast, Raeth-knight et al. (2007) and Weiss et al. (2008) found no effect of the propionibacteria strains on

milk production in the 12-week and 17-week trials ($P > 0.05$), respectively. Especially, Stein et al. (2006) and Lehloenya et al. (2008b) found positive effects of the administration of the strain P169 on milk production compared with the control group ($P < 0.05$). The F : C ratio of the diets in the trials above are similar, which is 40 : 60 for stein et al. (2006), 46: 54 for Lehloenya et al. (2008b) and 53.5 : 46.5 for Weiss et al. (2008), all of which showed lower forage proportion in the diet than this study (**Paper III**). However, the strain (P169) used in these three trials above is different from this study (T159) (**Paper III**). It is possible that if we increase the length of the period in this study (e.g. longer than 32 weeks) and increase the ratio of concentrate in the diet, it would be possible to observe the positive effect on the milk production by the administration of strain T159 in the lactating cows.

Based on the reports (Table 4.4) above, the effect of the propionibacteria on the milk production is unstable and complicated in different studies. In the future, the principle of fermentation patterns of different propionibacteria strains in the rumen needs to be explored by the trials in different doses of the strains in a long-period administration.

5. CONCLUSIONS AND FUTURE PERSPECTIVE

The main conclusions from the present work are:

Propionibacterium thoenii strain T159 could stimulate the substrate degradation and substantially inhibit methane formation using a typical diet for dairy cows *in vitro* (Chen et al., 2020a).

However, the strain T159 was unable to improve the rumen fermentation pattern, and therefore cannot decrease enteric methane emission and improve milk production in dairy cow at a high feed intake level *in vivo* (**Paper III**). Specifically, the results in the trial *in vitro* showed the increased ruminal propionate proportion and dry matter degradation in the incubation after 24 hours (Chen et al., 2020a), however, the identical effect could not be observed in a 3-week administration by the strain T159 *in vivo* (**Paper III**).

Still promising was that the strain T159 was able to persist or stimulate the growth or reproduction of the indigenous propionibacteria in the rumen for at least five days in the rumen of dairy cows at high feed intake (Chen et al., 2020b). The pretreatment effects observed in the 3-week animal experiments *in vivo* (**Paper III**) may confirm this conclusion.

The comparison with the previous literature suggested that the observed effect of propionibacteria on methane

emission and milk production was independent both from the species and the doses of propionibacteria administered. Still, the duration of the administration and the combination of several probiotics (including propionibacteria) could have an effect, by the administration of single or combined probiotics longer than 6 weeks showing partly positive effects on milk production.

Additionally, the comparison with the previous studies suggested that the observed effect of propionibacteria on methane emission and/or milk production might be independent from the ruminant species, the feed intake levels and the F : C ratios of the diet.

For the future perspective, the C^{14}-labeled substrates technique may be used to identify the different sources of the propionate in the rumen (Sutton et al., 2003). More explorations could be accomplished to identify the propionate produced by diverse ruminal propionate produces by C^{14}-labeled substrates, so that we could understand the pathway of propionate production by diverse propionate or succinate producers in the rumen better.

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7. PAPERS (I-III)

Paper I

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Propionic acid bacteria enhance ruminal feed degradation and reduce methane production *in vitro*

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Propionic acid bacteria enhance ruminal feed degradation and reduce methane production *in vitro*

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ABSTRACT

Thirty-one strains of propionic acid bacteria were screened for their effects on methane production and volatile fatty acid concentrations using *in vitro* assays of rumen fluid from Norwegian dairy cows and a grass silage–concentrate mixture as substrate. Nine of 31 strains were further analysed for effects on substrate degradation. Propionic acid bacteria led to reductions of up to 20% in methane production. Seven strains stimulated volatile fatty acid production, and in their presence *in vitro* substrate degradation tended to increase ($P < .10$). Most consistent results were found with *Propionibacterium thoenii* T159, which reduced methane production by 20% and caused 8% and 21% overall increases in substrate degradation and total volatile acid production, respectively ($P < .05$). Concomitant beneficial effects of a reduction in methane emissions and an increase in feed degradation suggest that this strain may be a promising tool for improving the productive performance of dairy cows.

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Introduction

The rumen microbiome, which ferments the feed and thereby provides fermentation end products that can be utilized by the host animal, is crucial in ruminant digestion. Accordingly, measures that aim at improving feed utilization and animal productivity by feeding live microbes to ruminants has a long tradition (Nocek & Kautz, 2006; McAllister et al., 2011). A major goal is to reduce enteric methane formation during rumen fermentation. Methane production represents an energy loss for the ruminant constituting 3 to 10% of its gross-energy intake (Niu et al. 2018). Moreover, methane is an important greenhouse gas and about 17% of all anthropogenic methane released into the atmosphere originates from domestic ruminants (Knapp et al. 2014). Methane is produced by methanogens from CO_2 and hydrogen formed during the microbial fermentation of the feed into volatile fatty acids (VFA) (Boadi et al., 2004). Acetate, propionate and butyrate are the major VFA produced that enter the bloodstream and serve as a source of energy and as a substrate for anabolic functions in ruminants. The propionate fermentation pathway is distinguished from the pathways leading to acetate and butyrate by not liberating hydrogen (Boadi et al., 2004). Hence, a positive correlation between enteric methane production and the ratio of ruminal acetate to propionate has been established (Russell, 1998). Therefore, stimulating propionate fermentation in ruminants might lower methane emissions. Moreover, unlike acetate and butyrate, propionate is a gluconeogenic VFA and thus can increase the availability of energy to the mammary gland (Yost & Young, 1977; Zárate, 2012). Propionate is an end product of the fermentation of various bacterial species, including the organisms of the family *Propionibacteriaceae*. Fed to dairy cows, strains of *propionic acid bacteria* (PAB) have been reported to have positive effects on production, including increased propionate levels in the rumen and improved milk yield (Stein et al., 2006; Adams et al. 2008; de Ondarza & Seymour, 2008; Weiss et al., 2008). Vyas et al. (2014) and Jeyanathan et al. (2019) studied the effects of *Propionibacterium* on methane production in ruminants. None of the strains tested was found to affect the total methane production. However, Vyas et al. (2014) observed that the feed intake was higher in the beef heifers fed *Propionibacterium* and the ratio between the methane produced and the feed consumed was lower by 8% to 13%. Other PAB strains were studied using *in vitro* experiments on rumen fluid from Canadian beef cattle (Alazzeh et al., 2013). That work showed that

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three PAB strains could reduce methane formation *in vitro* by 7 to 15%, yielding decreased proportions of acetate, and/or increased propionate proportions or yielding no change in the proportion of these VFA.

In this work, using rumen fluid from Norwegian Red dairy cows, we studied a large number of PAB isolates for their ability to affect the production of methane and of VFA and the ruminal feed degradation *in vitro*.

Materials and methods

Bacterial strains and growth conditions

To search for strains that could favourably alter rumen metabolism, we initially screened a total of 149 PAB strains for their effects on methane and gas production *in vitro* (data not shown). Most of the strains from the initial screening had no effect on methane production. Thirty-one of the 149 PAB strains chosen for the present study (28 strains of the genus *Propionibacterium*, 2 strains of *Tessaracoccus* and 1 strain of *Luteococcus*, Tables 2 and 3) all showed inhibitory effects on methane production. We conducted additional *in vitro* experiments with 9 promising strains out of the 31, in which feed degradation was measured as the weight difference before and after incubation in the rumen fluid. The PAB strains used in this study were isolated from Norwegian Red raw milk. These strains have been shown to produce propionic acid from lactic acid under anaerobic conditions (Holo et al., 2002). The strains were grown anaerobically at 37 °C in 10 mL screw capped glass tubes filled with sodium lactate broth (SLB) containing 10% tryptone, 10% yeast extract, 1.2% sodium lactate and 0.25 g of K_2HPO_4 per litre (Malik et al., 1968). Cultures to be used in the *in vitro* experiments were inoculated with 100 µL of exponentially growing cells and incubated for two days before used in the experiments. Viable counts of PAB were determined after anaerobic incubation at 37 °C on solidified SLB that contained 1.5% agar. For the determination of viable PAB in samples from the *in vitro* experiments, the agar plates were supplemented with metronidazole (4 µg/mL) (Jan et al., 2002).

Feed and rumen fluid

A feed mixture whose composition was comparable to that used for feeding Norwegian Red dairy cows in peak lactation was used as the substrate for the *in vitro* incubations (Table 1). The feed dry matter (DM) used in the *in vitro* experiments contained 60% grass silage and 40% concentrate milled to pass 4 mm and 1 mm screens, respectively. The concentrate DM contained 40% barley, 40% wheat and 20% soybean meal.

Table 1. Ingredients and chemical composition of the substrate (% dry matter, DM) used in the *in vitro* experiments 1 to 6.

Item	% DM
Ingredients	
Grass silage	60
Barley	16
Wheat	16
Soybean meal	8
Chemical composition	
Crude protein	16.5
Acid detergent fibre	27.1
Neutral detergent fibre	43.4
Non-fibrous carbohydrates	33.8

Rumen fluid was obtained from two non-lactating Norwegian Red cows, both fitted with a permanent ruminal cannula. The cows were kept in a metabolism unit authorized by the Norwegian Animal Research Authority and fed a standardized diet consisting of grass and concentrate that met maintenance requirement. The concentrate mixture contained approximately 180 g crude protein (CP)/kg DM and 120 g neutral detergent fibre (NDF)/kg DM. Rations of hay and concentrate were offered in equal meals at 06.30 h and 14.30 h. Samples of rumen fluid from the two cows were taken through the fistulae two hours after morning feeding, filtered through two layers of cheese cloth, mixed in a ratio of 1:1, flushed with CO_2 and then, within 30 minutes, mixed with two parts of the buffer prepared according to Menke et al. (1979). This freshly prepared buffered rumen fluid was used for the *in vitro* experiments.

In vitro experiments and sampling

During initial screening and in subsequent experiments 1 and 2, 10 mL of buffered rumen fluid (Menke et al., 1979) was mixed with 100 µL of the PAB culture (2×10^8 to 4×10^8 colony forming units, CFU, in total) and 50 mg of feed in 25 mL serum bottles under a stream of CO_2. The bottles were sealed with rubber serum stoppers and aluminium crimp caps and incubated for 24 h at 39°C. The control samples were supplemented with 100 µL of sterile SLB instead of PAB culture. Experiments 3, 4, 5 and 6 were conducted as described above, except that the incubations were carried out in stoppered 125 mL serum bottles using 450 µL of the PAB culture (or 450 µL SLB), 45 mL of buffered rumen fluid and 500 mg of feed. During screening and in experiments 3 to 6, all treatments and controls were carried out in triplicate. In experiments 1 and 2, treatments were carried out in triplicate and control samples were replicated 9 times.

The pressure in the vials was measured after 24 h of incubation using a DPG-200 Digital Pressure Gauge (Dwyer Instruments, Inc., Michigan City, IN). Gas samples were taken from the headspace of the vials

with a gas tight syringe and the methane concentration was measured using a gas chromatograph (Model 7890A, Agilent, Santa Clara, CA, US) that had a 20-m wide-bore Poraplot Q (0.53 mm) column at 38°C with back flushing and He as the carrier gas. The methane production (V_{CH4}, mL) was calculated as:

$$V_{CH4} = \text{ppm}_{CH4} \times V_{HS} \times 10^{-6} \times \Delta P$$

where V_{HS} is the volume of the vial headspace (mL) and ΔP is the vial pressure relative to standard pressure (atm).

Liquid samples for the analyses of the VFA concentrations were stored at −20°C. To determine the feed degradation in the 9 strains in experiments 3, 4, 5 and 6, the contents of the rinsed bottles were centrifuged at 7000 × *g* for 10 min in 50 mL of pre-weighed polypropylene centrifuge tubes. The supernatants were discarded, and the pellets were washed twice with distilled water and oven dried at 65°C for 7 days and weighed. The amount of feed DM that had been digested was calculated as the difference in weight between the pellets from samples taken at the start and the end of the incubation.

Chemical analysis

The contents of CP, acid detergent fibre (ADF), NDF and non-fibrous carbohydrates (NFC) in mixed feed DM were determined by Dairy One Forage Laboratory (Ithaca, NY) using near-infrared reflectance spectroscopy (Table 1).

The methane concentrations of the gas samples were determined using a flame ionization detector calibrated to certified standards of 2, 100 and 10,000 ppmv (Yara, Norway).

For the VFA analysis, liquid samples were thawed and 5 mL aliquots were mixed with 0.3 mL of 50% H_2SO_4 and 15 g of sodium sulphate and extracted with 25 mL of diethyl ether. Samples from the ether phase were analysed for VFA by gas chromatography using a Perkin Elmer Autosystem GC equipped with a flame ionization detector and a Supelco 2 m, 0.635 cm OD and a 2 mm ID glass column, packed with GP 10% SP 1000/1% H_3PO_4 on a 100/120 Chromosorb WAW (Kraggerud et al., 2014). The injection temperature was 210°C and the carrier gas was nitrogen at 40 mL/min. The following temperature programme was applied: 120°C for 1 min and then 15°C/min to 190°C for 5 min.

Statistical analysis

The results were analysed using a one-way analysis of variance (ANOVA), with the strain as the fixed factor and the vial as the random factor. Then Fisher's least significant difference (LSD) post hoc test was conducted using a 95% confidence interval (Minitab version 19 from Minitab Inc., PA, USA).

Results and discussion

Effects on production of volatile fatty acids and methane

The control samples in experiments 1 and 2 produced 48 mL and 51 mL methane per g of feed DM, respectively (Tables 2 and 3). Most of the PAB strains tested did not cause a significant change in methane production, which ranged between 42 and 54 mL/g of feed DM. However, incubation with *P. jensenii* LMGT2826 and

Table 2. Effect of propionic acid bacterial strains *Propionibacterium* (*P.*) and *Tessaracoccus* (*T.*) on production of methane and volatile fatty acids in experiment 1.

Bacterium added	Strain	Total VFA[a], mM	Acetate, mM/ 100 mM total VFA	Propionate, mM/ 100 mM total VFA	Butyrate, mM/ 100 mM total VFA	Acetate: propionate	Methane, mL/g DM
P. sp.	LMGT2789	77.0	68.9	16.8	14.2	4.09	48.1
P. acidipropionicii	LMGT2831	75.4	68.6	17.2	14.2	4.00	49.7
P. freudenreichii	LMGT2832	76.1	67.9	18.0*	14.1	3.78*	43.9
P. freudenreichii	LMGT2833	77.1	68.7	17.1	14.2	4.02	47.1
P. freudenreichii	LMGT2842	72.4	67.6*	17.6	14.7	3.84	55.0
P. jensenii	LMGT2864	71.7	67.6*	17.3	15.1*	3.90	47.3
P. sp.	T1	76.2	68.6	17.1	14.3	4.01	47.5
P. sp.	T25	78.7	69.3	17.1	13.7	4.06	49.0
P. freudenreichii	T28	80.4*	69.6	16.9	13.5	4.12	50.1
P. freudenreichii	T30	73.0	67.9	16.8	15.3*	4.05	47.1
P. freudenreichii	T62	79.6*	69.1	16.9	14.0	4.08	47.8
P. sp.	T88	81.2*	69.8*	17.0	13.2*	4.11	52.1
T. bendingoniensis	T93	71.9	67.5*	17.6	14.9	3.83	48.4
T. bendingoniensis	T104	77.2	69.3	17.0	13.7	4.09	48.6
P. freudenreichii	T114	82.0*	69.4	17.1	13.5	4.06	53.7
P. acidipropionicii	T122	77.2	69.2	17.3	13.5	4.00	45.2
None	Control	74.1	68.7	17.1	14.2	4.01	47.9
	SEM	2.01	0.36	0.24	0.35	0.06	0.68

Mean values with an asterisk differ significantly ($P < .05$) from control.
[a]Sum of acetate, propionate and butyrate.

Table 3. Effect of propionic acid bacterial strains *Propionibacterium* (*P.*) and *Luteococcus* (*L.*) on production of methane and volatile fatty acids in experiment 2.

Bacterium added	Strain	Total VFA[a], mM	Acetate, mM/100 mM total VFA	Propionate, mM/100 mM total VFA	Butyrate, mM/100 mM total VFA	Acetate: propionate	Methane, mL/g DM
P. jensenii	LMGT2816	79.4	72.8	15.9	11.3*	4.57	46.6
P. jensenii	LMGT2822	73.6	72.5	16.0	11.5	4.52	44.6
P. jensenii	LMGT2823	73.4	71.8	16.2	12.1	4.44	46.8
P. jensenii	LMGT2824	73.8	71.6	16.2	12.2	4.41	49.7
P. jensenii	LMGT2825	71.3	71.2	16.4	12.4	4.35	47.9
P. jensenii	LMGT2826	75.5	72.4	16.3	11.4*	4.45	41.7*
P. thoenii	LMGT2827	74.1	71.5	16.2	12.2	4.41	47.0*
P. freudenreichii	LMGT2841	75.0	72.2	16.1	11.7	4.49	47.3
P. sp.	T22	71.4	71.1	16.4*	12.4	4.33*	47.4
P. sp.	T24	85.6*	71.9	16.1	12.0	4.51	43.3
P. freudenreichii	T27	73.1	72.2	16.0	11.9	4.52	45.3
P. freudenreichii	T31	81.4	72.9	15.9	11.2*	4.57	44.5
P. propinicus	T83	85.9*	74.1*	15.2	10.7*	4.88*	43.9
P. sp.	T88	80.3	72.4	15.8	11.8	4.58	41.7
L. japonicus	T145	75.8	72.4	15.9	11.7	4.54	47.8
P. thoenii	T159	89.6*	72.5	16.7*	10.8*	4.34	40.7*
None	Control	74.0	72.0	15.8	12.2	4.57	51.0
	SEM	2.53	0.37	0.22	0.25	0.08	0.42

Mean values with an asterisk differ significantly ($P < .05$) from control.
[a]Sum of acetate, propionate and butyrate.

P. thoenii LMGT2827 or T159 resulted in reductions ($P < .048$) in methane production of 18%, 8% and 20%, respectively, relative to the control samples. In previous reports, the effects of *Propionibacterium* had been found in experiments with dairy cattle, in particular using the *Propionibacterium acidipropionici* strain P169, which may increase the efficiency of milk production (de Ondarza & Seymour, 2008; Weiss et al., 2008), but other strains tested did not show this effect (Seo et al., 2010). Using rumen fluid from cattle, it was shown that several strains of PAB could reduce ruminal methane production *in vitro* (Alazzeh et al, 2013), but this effect was not seen with strain P169 (Alazzeh et al., 2014). This finding conflicts with that of Stein et al. (2006); they found that the strain P169 caused higher rumen propionate levels *in vivo*.

The total VFA concentrations (the sum of acetate, propionate and butyrate concentrations) in the controls at 74 mM were very similar in experiments 1 and 2 (Tables 2 and 3). None of the PAB strains caused a significant reduction in total VFA production, but seven strains had stimulatory effects ($P \leq .046$). Relative to the controls, the strain T159 showed the strongest stimulation of VFA production, increasing levels by 21% ($P = .001$), with the proportion of propionate being higher by 6%, but without a change in acetate. The presence of four (T28, T62, T114 or T24) of the seven stimulatory strains did not change the proportions of acetate, propionate and butyrate. With the addition of the strain T83, the proportion of acetate increased slightly compared to the controls ($P < .001$), leading to a higher ratio of acetate to propionate ($P = .011$). The strain T88 was tested in both experiments and it increased ($P \leq .024$) VFA production by about 10% and the proportion of acetate by 2% in experiment 1 (Table 2) but did not do so in experiment 2 (Table 3), relative to the controls. The majority of the 31 strains had no effect on the proportions of butyrate; only T88, T83 and T159 led to a slight decrease in proportion of butyrate compared to that in the controls ($P \leq .038$).

While the dominating VFA in the rumen is acetate, the metabolic end products of the PAB that grow on sugars or lactate are propionate and acetate in a ratio of 2:1 or higher. Previous studies found that PAB strains could increase rumen propionate levels (as a proportion of the total VFA) and lower levels of acetate *in vivo* (Stein et al., 2006; Raeth-Knight et al., 2007; Weiss et al., 2008) and *in vitro* (Akay & Dado, 2001; Alazzeh et al., 2013), suggesting that PAB could significantly contribute to rumen fermentation. Luo et al. (2017) showed that PAB can degrade lactate in the rumen fluid *in vitro*. In experiments 1 and 2, the molar ratios of acetate to propionate in the controls were 4.0 and 4.6, respectively. Slightly lowered acetate to propionate ratios ($P \leq .048$) were observed using the strains *P. freudenreichii* LMGT2832 and *P.* sp. T22 than in the controls.

Including metronidazole in the SLB agar plates (Jan et al., 2002) enabled us to study the fate of the PAB after 24 h of incubation. With no PAB in the sample, we found a viable count of 1×10^6 CFU per mL on this medium in the buffered rumen fluid, and this number increased about two-fold over 24 h in the control vials without PAB (data not shown). None of the PAB strains added to the vials exhibited growth in the bottles during incubation, and their viable count decreased by 17% (the strain LMGT2826) to 95% (the strain LMGT2827) (results not shown). Although we observed a

reduction in the number of PAB cells during incubation, we cannot rule out the initial growth of the PAB followed by a death phase during the 24 h of incubation. However, PAB grow slowly; using published data on the maximal specific propionate production and the growth rate (Lee et al., 1974), we calculated that the average rate of propionate synthesis in our experiments would be lower than the observed stimulation, compared to the controls.

Thus, most of the propionate in our experiments must have been produced by other organisms in the rumen samples. For the same reason, it seems unlikely that the increase in propionate observed when feeding P169 is produced by the strain P169 itself, since the recovery for this strain was 10^6 CFU per mL or lower (Peng et al., 2011). However, Luo et al. (2017) showed that propionate can be formed by PAB at high rates and we cannot rule out that P169 could have produced propionate more rapidly *in vivo* than *in vitro*, as a result of the stimulation of natural microbial inhabitants of the rumen.

Our results with rumen liquid from Norwegian Red dairy cattle differ somewhat from those in previous reports with respect to ruminal VFA proportions. Two strains led to an increase in acetate proportions but not to a concomitant increase in methane yield. Three other strains tested in the present study caused increases in the propionate but no change in the acetate proportions or the methane yield, except for *P. thoenii* T159, which led to a 20% reduction in the methane yield. This shows that the PAB strains *in vitro* were able to partially redirect the carbon flow. Moreover, the PAB strains LMGT2826, LMGT2827 and T159 were even able to redirect it away from methanogenesis.

The data indicate that although propionate fermentation is stimulated, acetogenesis, possibly homoacetogenesis (Joblin, 1999), appears to be stimulated even more in some cases. Methane production in our experiments was 40.7 to 55.0 mL/g DM, a little higher than reported for Canadian cattle (Alazzeh et al., 2013; Alazzeh et al., 2014). In our study, the methane production corresponded to about 0.1 mmol C in the methane per vial. At most, this was reduced compared to the controls by 0.02 mmol C (the strain T159), while the observed increase in VFA production with this strain corresponded to 0.35 mmol C. Thus, our results cannot be explained merely as the redirection of the carbon flow away from methanogenesis to VFA production. Rather, the data indicate increased feed degradation and fermentation through pathways that produce mainly acetate and, to a lesser degree, propionate. They also indicate that PAB strains can stimulate metabolism of other microbes in the rumen.

The PAB have been shown to affect the growth of a wide variety of microbes, including bacteria, yeasts and moulds. Most studies have focused on selectively inhibiting the growth of microbes (Holo et al., 2002; Lind et al., 2007; Schwenninger et al., 2008; Faye et al., 2011), but the PAB's properties of stimulating growth are also known (Kaneko, 1999; Jan et al., 2002; Warminska-Radyko et al., 2002); 1,4-dihydroxy-2-naphthoic acid secreted by PAB can stimulate the growth of *Bifidobacteria* and various other anaerobes (Kaneko, 1999; Fenn et al., 2017). In line with this, it was reported that *P. acidipropionicii* P169 could stimulate rumen feed degradation (Sanchez et al., 2014).

Feed degradation

In the controls, degradation, that is, the amount of substrate digested after 24 h ranged from 57% to 63% (Table 4). The DM degradation varied greatly when the PAB strains were added, except for the strains LMGT2825 and LMGT2841. With these two particular strains, we saw no stimulation of degradation. Each of the other strains increased feed degradation ($P < .05$) in at least one of the four experiments. In experiment 5, several strains increased degradation to about 72%.

Table 4. Effect of propionic acid bacterial strains *Propionibacterium* (*P.*) and *Tessaracoccus* (*T.*) on substrate degradation in experiments (Exp.) 3 to 6.

Bacterium added	Strain	Substrate degradation (% dry matter)				
		Exp. 3	Exp. 4	Exp. 5	Exp. 6	Average
P. jensenii	LMGT2816	60.9	59.2	64.4	71.3*	63.9
P. jensenii	LMGT2824	54.6*	60.2	57.0	69.7*	60.4
P. jensenii	LMGT2825	59.6	59.6	60.5	57.8	59.4
P. freudenreichii	LMGT2841	59.0	60.6	63.5	60.5	60.9
P. sp.	T24	58.5	60.5	72.3*	60.4	63.0
P. freudenreichii	T31	58.9	59.5	72.4*	62.6	63.3
P. sp.	T88	61.3	58.6	71.3*	63.2	63.6
T. bendingoniensis	T93	61.1	59.2	69.2*	64.7	63.5
P. thoenii	T159	57.2	63.3*	71.7*	67.5	64.9*
None	Control	56.5	59.4	61.0	63.2	60.0
	SEM	0.55	0.32	1.11	0.82	1.97

Mean values within a column with an asterisk differ significantly ($P < .05$) from control.

Only one strain, LMGT2824 in experiment 3, resulted in a slight (4%) reduction in feed degradation.

The strongest overall stimulation ($P < .008$) was obtained with strain T159, with an average increase in feed degradation of 8% compared to the controls. The strains LMGT2816, T88 and T93 showed the same trend ($P \leq .057$), with an average increase in degradation of 6%. The beneficial effects of PAB on ruminal feed degradation are not well understood. The PAB might stimulate the growth and activities of ruminal microorganisms by providing growth factors, e.g. 1,4-dihydroxy-2-naphthoic acid (Kaneko, 1999; Fenn et al., 2017).

The variability in feed degradation across experiments was greater in incubations with PAB than in the controls, ranging from no effect to 12% increase. This may reflect day-to-day differences in the rumen microbiome or in the condition of the PAB cultures used.

We have found that PAB can increase feed degradation. This may be a general property of PAB, but the strain T159 showed the most consistent results. Interestingly, the same strain also showed the strongest effects in the *in vitro* test with rumen fluid from Canadian beef cattle (Alazzeh et al. 2013). In those experiments, with different feed, the effects on methane yield were smaller, although propionate was stimulated at the expense of acetate. It is noteworthy that the strain T159 influenced the metabolism of rumen microbiota that were likely quite different in our and Alazzeh et al.'s (2013) studies, as evidenced by the differences in the ruminal acetate to propionate ratios. Such differences and the different feeds used may have contributed to the somewhat different outcomes in the two studies.

Using a diet typical for dairy cows, we have shown that *P. thoenii* T159 can stimulate feed degradation and inhibit methane formation at the same time. These two beneficial traits add the strain T159 to the list of promising direct-fed microbials for more efficient feed utilization by ruminants, but this has to be evaluated *in vivo*.

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Paper II

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BRIEF REPORT

Ruminal survival of *Propionibacterium thoenii* T159 in dairy cows at high feed intake

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ABSTRACT

Propionibacterium thoenii strain T159 (5×10^{11} CFU) were administered daily in the rumen of four Norwegian Red dairy cows. Total *Propionibacterium* in the rumen were substantially increased during and after the treatment with T159 relative to the background. Strain T159 was able to persist for at least five days in the rumen of dairy cows at high dry matter intake (3.9% of body weight).

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KEYWORDS
Propionic acid bacteria; direct-fed microorganisms; Norwegian Red; ruminant; ruminal persistence

Introduction

Propionic acid bacteria (PAB) produce propionic acid as a by-product of (feed) fermentation. PAB isolated from dairy products have been applied as direct-fed microorganisms (DFM) for ruminants to increase ruminal propionate proportions (Stein et al., 2006; Weiss et al., 2008). DFM can cause a shift in rumen fermentation from acetate to propionate synthesis. This glucogenic volatile fatty acid can improve the energy available to the ruminant and thus its production performance (Stein et al., 2006; Vyas et al. 2016). In addition, DFM also have the potential to reduce H_2 generation and thus reduce enteric methane production. *Propionibacterium acidipropionici* strain P169 and *P. thoenii* strain T159 are among the most promising DFM in terms of ruminal survival and rumen fermentation effects. In beef heifers it was found that administered strain P169 (Vyas et al., 2014) and T159 (Vyas et al., 2016) could persist for 9 h at elevated levels in the rumen. However, ruminal DFM detectability beyond this time point was not studied. Establishment and ruminal survival of PAB is likely influenced by feeding level. The objective of this work was to determine the ability of *Propionibacterium thoenii* strain T159 to persist for several days in the rumen of dairy cows at high feed intake.

Materials and methods

The experiment was authorized by the Norwegian Animal Research Authority. Four multiparous, rumen-cannulated Norwegian Red cows were initially 35 ± 18 days in milk (mean ± SD), had a body weight of 558 ± 29 kg and yielded 32.9 ± 7.2 kg milk daily. The cows were housed in tie-stall, had free access to water and were milked at 0730 and 1930 h. Cows were allowed <1% feed refusal and offered daily 8.6 kg dry matter (DM) of concentrate (in g/kg DM; crude protein, 223, NDF, 180, starch, 337) and 13 kg DM of grass silage (in g/kg DM; NDF, 539, CP, 147). The feed was divided into three equal portions and provided at 07.00, 14.00 and 21.00 h. The experiment lasted 15 days and consisted of three sampling periods: pre-treatment (control), treatment and post treatment. Samples of rumen content were collected through the rumen cannula. Control samples were collected at days −2, −1 and 0. PAB were administered through the rumen cannula at days 0–7 as detailed below. At 3 and 24 h after PAB treatment, samples were collected at days 1–8, and post treatment samples at days 9–12.

Propionibacterium thoenii T159 was grown anaerobically at 30°C for 48 h in sodium lactate broth (SLB) liquid medium (Malik et al., 1968). Five hundred mL of T159 liquid culture (1×10^9 CFU/mL) was administered daily at 13.50 h to each cow through the rumen cannula. On the first day of treatment (day 0), all cows were administered with an initial dose of 1.0 L culture. Rumen content (mixture of fluid and solid material) was collected through the rumen cannula at 13.40 h during the control period and at 13.40 and 17.00 h subsequently. A total of one liter of rumen content was

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collected at 5 depths from the ventral to the dorsal rumen. The sample was thoroughly mixed, and 50 mL of rumen content was transferred into plastic tubes and immediately frozen at −18°C.

The levels of rumen *Propionibacterium* spp. were analyzed by using polymerase chain reaction (PCR) with primers targeting their 16S rRNA gene. The thawed samples were processed in a Stomacher 400 Circulator (Seward, UK) to detach microbes from solid rumen contents. After filtration with 2 layers of cheese cloth, 100 µL aliquots were transferred to 1.5 mL centrifuge tubes and subjected to microwave treatment at 800 W for 1 min. The DNA was isolated using a NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel) according to the manufacturer's instructions with the following modifications: The microwave treated samples were mixed with 200 µL of the chaotropic salt solution. The mixtures were left at room temperature for 1 min, then centrifuged at 10,000 × g for 1 min and the supernatants were applied to silica filter columns. After the washing steps DNA was eluted in 30 µL. Serial dilutions of the DNA were made in water and served as template in PCR with OneTaq polymerase (New England Biolabs, Massachusetts, USA) using primers PAB1 (5′ – TAG GGT GCG AGC GTT GTC CG – 3′) and PAB4 (5′ – GAA CCG CCT TCG CCA CTG GT – 3′) at 0.2 µM, 1 µL DNA and 0.25 µL polymerase in 50 µL reactions. The following protocol was used: 5 min initial denaturation at 94°C followed by 35 cycles of denaturation (15 s at 94°C), annealing (30 s at 68°C), and extension (5 min at 68°C). PCR products were analyzed by electrophoresis of 10 µL aliquots in 2% agarose gels with 0.004% peqGREENTM (VWR International, Lutterworth, UK) and visualized by UV light. The level of PAB was calculated from the highest dilution of template DNA giving a detectable PCR product and related to DNA from a culture of T159 of known cell density. Microwave treatment has been shown to release DNA from various bacteria (Bollet et al., 1991), but our microwave treated rumen samples contained unknown substances inhibiting the PCR reaction and no PCR product was obtained even from the samples taken after administration of T159. We therefore introduced a simple cleanup procedure using silica. A centrifugation step was included to remove insoluble material. The sensitivity of this analysis was compared by colony counting on SLB agar. The results showed that using the DNA isolation procedure described we were able to detect 10^4 CFU/mL (corresponding to 30 CFU per reaction) of strain T159 by PCR.

Means of PAB concentrations were compared between treatment and post treatment period by Student's paired *t*-tests using Minitab (19, Minitab Inc., PA, USA). Significance level was set to $P < .05$.

Results and discussion

Before administration of T159, the PCR analysis indicated a background level of PAB of 10^4 CFU/mL in the rumen content control samples (day −2, −1, 0) of all cows, which is consistent with the results from Davison (1998). This low background allowed us to investigate the persistence of the administered T159. In the studies in beef heifers (Vyas et al., 2014, 2016) and dairy cows (Jeyanathan et al., 2019), the background PAB numbers were much higher and could complicate the determination of PAB persistence. The addition of T159 into the rumen caused a strong increase in the concentration of PAB in ruminal content (Figure 1). The increase was about 2 logs, close to the value expected from the dose given, and was observed already after the first administration. We did not detect any difference in PAB numbers between samples taken at 3 and 24 h after T159 administration (results not shown). Daily administration of T159 for the consecutive 8 d of the treatment period did not cause a notable further increase in the PAB level.

The concentration of PAB in the ruminal content remained high during the entire 5 days after the last treatment (Figure 1). For three of the cows (5979, 6043 and 6260), with ≤1% feed DM refusals, only a weak decline ($P < .034$) in PAB content was seen. A stronger reduction in ruminal PAB concentration was observed with cow 6322, the cow which had only an average total DMI of 17.7 and 19.1 kg per day in the treatment and posttreatment period, respectively. The reason for the feed refusal (grass silage) remains unknown, but it may have led to unfavorable conditions in the rumen for PAB maintenance or

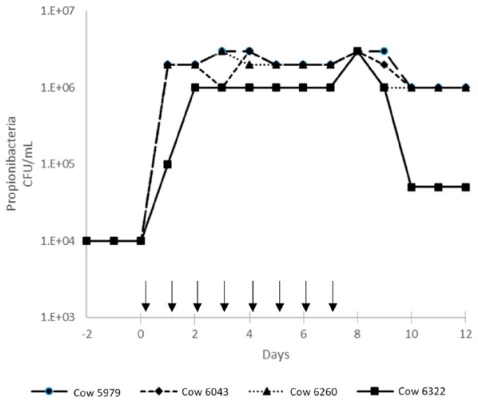

Figure 1. Kinetics of propionibacteria concentrations in the rumen of four cows. *Propionibacterium thoenii* strain T159 was administered through the rumen cannula on days 0–7 (indicated by arrows). Samples at days 1–8 were taken 24 h after administration of T159.

growth. The results of the present study demonstrated a comparatively long persistence of T159 in the rumen over at least 5 days post treatment. Previous work showed that T159 was detectable 9 h post treatment in the rumen of beef heifers (Vyas et al., 2016), but it was unclear if T159 can survive beyond 9 h and at high feed intake in the dairy cow rumen. The feeding level (DMI, % of body weight) in our study was about twice as high as in other reports administrating PAB to beef heifers (Vyas et al., 2014, 2016) and dairy cows (Jeyanathan et al., 2019). The rumen outflow rates of liquid and particles increase with increasing DM intake (Volden et al., 1998; Volden, 1999). Assuming a short rumen retention time due to the high feeding level, the low rate of disappearance from the rumen is a clear indication that the administrated PAB can grow in the rumen environment of dairy cows at high feeding level. At a high feeding level, the inoculant retention time is shorter and the supply of substrates for growth can be better, both factors favoring fast but not slow growing bacteria. Based on experiments comparable to the present study, rumen liquid and particulate outflow rates are expected to be between 15–20 and 8–10%/h, respectively (Volden et al., 1998; Volden, 1999). Our data show that the inoculated strain T159 was able to grow in the rumen faster than the outflow rate. With no growth the level of *Propionibacterium* would have been reduced to background levels within 3 d. To be stably established in the rumen the inoculant would have to grow with a mean doubling time of 3–5 h (unattached to particulates) or 7–9 h (attached to particulates). Albeit in laboratory media, a doubling time of 5 h has been reported for *P. thoenii* (Paik & Glatz, 1997). In addition to the ruminal survival of T159 in the post treatment period, the strain T159 could have stimulated other PAB strains in the rumen leading to the detected elevated PAB concentrations.

The present work showed that *Propionibacterium thoenii* strain T159 was able to persist for at least five days in the rumen of dairy cows with high feed intake. Recently, T159 was found to be promising for a substantial reduction of methane emissions per unit DM *in vitro* (Chen et al., 2020), but not in beef heifers with low feed intake (Vyas et al., 2016). Experiments with dairy cows with high feed intake still have to confirm the methane mitigating effect of the direct-fed microbial *Propionibacterium thoenii* strain T159.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Paper III

The effect of *Propionibacterium thoenii* T159 on methane emission, ruminal characteristics, and milk production in dairy cows at high feed intake

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ABSTRACT

The objective of this study was to determine the efficacy of *Propionibacterium thoenii* T159 to mitigate enteric methane (CH_4) emissions, to improve the performance of Norwegian Red cows at high feed intake in a three-week administration. Two consecutive experiments were conducted with five intact dairy cows (experiment 1, exp1) and four ruminally cannulated dairy cows (experiment 2, exp2) fed the same basal diet consisting of 60% grass silage and 40% concentrate (dry matter basis). The cows in T159 group were living strain culture of T159 (8.5×10^{11} colony forming units/head × day), and the control cows were fed dead strain culture of T159. The dead or living strain culture of T159 were administered daily before feeding directly into the rumen (exp1: oesophageal tubing, exp2: through the rumen cannula). No differences ($P < 0.05$) between treatments in milk production, dry matter intake (DMI), methane production (g/d), methane yield (g/ kg DMI) and methane emission intensity (g/kg ECM) were observed (exp1, exp2). Milk composition slightly differed between treatments in exp2 only, with the milk lactose and urea content being lower ($P < 0.05$) in T159 group. In addition, we assessed nutrient digestibility and rumen fermentation variables in ruminally cannulated cows (exp2). Compared with the control group fed dead strain culture of T159, the living strain culture of T159 showed no impact on the digestibility of organic matter (OM) and neutral detergent fiber (NDF). Moreover, no treatment effects were observed in most

rumen fermentation parameters in both experiments except butyrate. The strain T159 elevated the molar proportion of ruminal butyrate (9.73 vs. 9.63 % of total short-chain fatty acids, SCFA, $P < 0.01$). In conclusion, the mitigation potential deficient of T159 on enteric methane emissions from dairy cows at high feed intake might be attributed to their incapability to compete with methanogens for hydrogen and promote the propionate production in the rumen. The absence of the improvement on milk production may due to the failure of the propionate stimulation in the rumen by T159 in a short-term administration. In conclusion, the strain T159 was not able to present a significant impact on the mitigation of enteric methane emission, rumen fermentation and the performance in a short-term administration. Therefore, the long-term administration could be necessary to evaluate the effect of T159 in the future.

Key words: Propionibacteria, dairy cow, milk production, methane, direct-fed microbials

INTRODUCTION

Enteric methane emissions from ruminants account for 17% of global methane emissions (Knapp et al., 2014), and 80% of the total methane emissions from livestock (Gill et al., 2010). Propionate synthesis and methane production was found to be inversely related in the trial *in vitro* (Russell et al., 1998). This is because propionate synthesis by propionibacteria (PB) requires electrons from hydrogen (H_2) and this reduction process has a

lower $\Delta G^{0'}$ compared to methanogenesis by archaea (Boadi et al., 2004). Furthermore, ruminal propionate is absorbed by the rumen epithelium (Lehloenya et al., 2008a) and transferred by the blood to the liver to synthesize glucose through gluconeogenesis (Stein et al., 2006), thereby improving the energy status, the milk production, and milk lactose contents of early lactating cows (Stein et al., 2006). As one of the indigenous inhabitants of the rumen, propionibacteria are facultative anaerobic actinobacteria in a ruminal population of 10^3~10^4 colony-forming units (CFU) per milliliter rumen fluid (Davidson, 1998), accounting for 1.4% of the total microbial population (Oshio et al., 1987). Probiotics in ruminant nutrition could ideally reduce enteric methane emission and improve the performance of wildlife and domestic animals. Chen et al. (2020a) and Alazzeh et al. (2013) showed that the addition of *Propionibacterium thoenii* strain T159 substantially reduced methane emissions per unit dry matter (DM) compared to control group (only SLB medium was added into the vial, with the same amount as the strain culture for treatment group), and to increase ruminal SCFA levels, propionate proportion and substrate degradation in the rumen fluid of the batch culture *in vitro*. However, Vyas et al. (2016) reported a failure of methane mitigation by the administration of T159 in beef cattle. Critically, there was no trial focused on the effect of T159 on feed digestibility and methane emissions in dairy cows until now. Recently, the strain T159 was observed to persist or stimulate the reproduction of indigenous

propionibacteria for at least 5 days in the rumen of dairy cows (Chen et al. 2020b). Therefore, we assume that the strain T159 could be a promising direct-fed microbial to mitigate enteric methane emission and improve the productive performance of dairy cows at high feed intake.

MATERIALS AND METHODS

Experimental Design

The experiment was authorized by the Norwegian Animal Research Authority. Two consecutive experiments with the same dietary treatments were conducted with lactating, multiparous Norwegian Red cows in the tied-stall metabolism unit belonging to the Faculty of Biosciences (BIOVIT) at NMBU. Experiment 1 was a 2 treatments × 4 periods cyclic change-over design with five intact cows (Table 1). Experiment 2 was a 2 treatments × 3 periods cyclical design with four rumen cannulated cows (Table 1). All the cows were allocated to two treatment groups in each period of both experiments. Dietary treatments included: (1) Control (fed the dead strain culture of T159), (2) *Propionibacterium thoenii* T159 (fed the living strain culture of T159). Each period lasted for twenty-one days, including sixteen days adaptation phase and five days sampling phase in exp1, and twelve days adaptation phase and nine days sampling phase in exp2.

Animals, Diet and Treatments

Five cows in their second lactation were recorded with an averaged (± SD) 58.2 ± 6.2 days in milk (DIM), a milk yield of 28.4 ± 4.726 kg/d, and a body weight (BW) of 569 ± 22.4 kg before exp1 started. Before the start of exp2, four ruminally cannulated donor cows, in their second, third, fourth and fifth lactation respectively, were recorded with an averaged 38.5 ± 13.4 DIM, a milk yield of 36.0 ± 3.50 kg/d, and a body weight of 646 ± 68.8 kg. The number of lactations of all the cows used in the trial are presented in Table 1. All the cows in exp2 were surgically fitted with a rumen cannula (ten centimeters in center diameter, Bar Diamond Inc., Parma, ID, USA) at the end of their first lactation.

In the treatment groups of both experiments, all the cows were fed with the basal diet and the living strain culture of T159 (8.5×10^{11} CFU/ head × day). The control cows were fed daily with the basal diet and the same amount of dead strain culture in both experiments.

The diet contained (on a dry matter basis) 60% silage and 40% concentrate. All the cows were offered three meals a day (0715, 1400 and 2200 h) in the trial. The dry matter content of the silage and concentrate was 32.8% and 92.3%, respectively. The grass silage was made from an organically grown first-cut mixed sward and harvest on the Frydenhaug from an organically grown meadow with a high clover content. The meadow was sown in the year 2010 and harvested for the first time in the year 2011. The grass silage was pressed into round bales. The ingredient

proportions and chemical composition of the silage and concentrate (Formel Basis 80, Felleskjøpet, Oslo) are presented in Table 2. All the cows had free access to water.

In both experiments, from day 1 to day 4 of the adaptation phase in each period, the diet was provided ad libitum with at least 10% leftovers. Feed residues were recorded daily at 0700 h. During the remaining days of the adaptation phase and in the sampling phase in each period, the diet offered was limited to correspond to 90% of the average intake to avoid residues and keep the ratio of silage : concentrate stable.

The Preparation and the Administration of Strain T159

The living strain culture of *Propionibacterium thoenii* T159 for the cows in T159 group was prepared according to Chen et al. (2020b). The dead strain culture for control cows was prepared by the sterilization of the living strain culture with high-pressure steam at 121.3 °C for 30 min, and then cooled down to 30 °C. The living or dead strain culture were administered daily in both groups before afternoon feeding at 14.00 h. On day 1 of each period, all the cows in both groups were administered with an initial double dose of one Liter (living or dead) strain culture and from day 2 to day 21 in each period, the dose was 500 mL daily. The living or dead strain culture was administered via esophageal tubing in intact cows (exp1, took approximately one to two minutes per cow) and directly into the rumen through the cannula in cannulated cows (exp2).

Collection of Data and Sampling

Gas Sample Collection

In both experiments, the individual enteric methane emission was measured on days 17, 18, 19, 20 and 21 of each period by sulfur hexafluoride (SF_6) tracer technique (Johnson et al., 1994). Fifteen days before the initial gas sampling, each cow was intraruminally dosed with a calibrated brass permeation tube of sulfur hexafluoride (mean ± SD, 2.53 ± 0.04 g; 12.5 × 40 mm) prepared by Agriculture and Agri-Food Canada (Semiarid Prairie Agricultural Research Centre, Saskatchewan, Canada). The predetermined average release rate of sulfur hexafluoride was 3.69 ± 0.177 mg/d. Representative breath samples of each cow were collected in pre-evacuated yokes (fixed on the shelf above each cow) over 24 h by means of capillary tubing fitted to a halter of the donor cow. Background air samples were collected over 24 h on the sampling days with two yokes and halter sets placed elsewhere in the barn to the height of the cow-head. Before collection, the yokes were washed twice with two bar (200 kPa) pure N_2. After sample collection, all the yokes were pressurized by one bar (100 kPa) pure N_2 and gas samples were collected after 45 min by a gas tight syringe. Then the gas samples in the syringe were injected into evacuated glass vials (13 mL) for analysis within 48 h. The gas samples were collected in triplicate per yoke.

Milk Yield Recording and Sampling

Cows were milked daily at 0730 and 1900 h and milk yield was recorded by Tru-test Milk Meter (Tru-Test Distributors, Ltd, New Zealand) twice daily on days 17 to 21 in exp1 and on days

12 to 21 in exp2. The milk samples for analysis from morning and evening milking on day 19 and day 21 in exp1, and on day 13 and day 15 in exp2 were collected. Proportional amounts from individual morning and evening milking of each day were mixed thoroughly and 40 mL per day per cow was conserved with a tablet of Bronopol (8 mg; 2 – bromo – 2 – nitropropane – 1, 3 – diol; D & F Control Systems, Inc., USA). The milk yield in exp2 was evaluated for days 12 to 16 only, because the subsequent feces and urine collection may have some effect on the milk yield.

Feed Sample Collection

Feed intake was recorded daily by weighing diets offered and refused (ort). In both experiments, diet samples were collected each day in week 2 and week 3 in each period. The silage samples were frozen at −20 °C and the concentrate samples were stored at room temperature. Samples were pooled per period and a subsample was dried at 60 °C and ground (Retsch ZM 100; Retsch GmbH, Haan, Germany) with a 1 mm screen before analysis.

Excreta Collection

In exp2, excreta were sampled in each period on days 19 to 21 (72 h). Feces were collected in steel trays located below the metabolism boxes. Urine was collected separately from feces with a funnel device attached around the vulva of the cows. The device diverts the urine by a hose into a plastic bucket with 500 mL 10% sulfuric acid (H_2SO_4). Feces and urine were weighed and sampled three times a day (0800, 1500 and 2100 h). Proportional amounts (10%) of respective fractions were pooled to one urine and one

fecal sample per cow per day and frozen at −20 °C before analysis. Subsamples of pooled feces were dried at 60 °C and ground (Retsch ZM 100; Retsch GmbH, Haan, Germany) with a 1 mm screen before analysis.

Rumen Sample Collection

In exp2, rumen fluid samples were collected through the rumen cannula on days 14 and 16 in each period. From each cow, 150 mL rumen fluid was collected from the ventral, central and dorsal rumen content by a syringe at 07.00 (before morning feeding), 07.30 (immediately after morning feeding), 08.00, 09.00, 10.00, 12.00, 14.00 (before afternoon feeding), 14.30 (immediately after afternoon feeding), 15.00, 16.00, 17.00, 19.00 and 21.00 h. The pH value of each sample was measured by a pH meter immediately. Then 9.5 mL of each sample was transferred to test tubes containing 0.5 mL formic acid (100% HCOOH). Afterwards, all the samples were stored at −20 ˚C until analysis.

Laboratory Analysis

Feed and Excreta

Feed and excreta were analyzed according to standard procedures (AOAC, 1995). Dry matter (DM) was determined by drying samples at 103.0 °C to a constant weight. Total ash was determined gravimetrically after pyrolysis at 550.0 °C for 4 h in a muffle furnace (Milestone Srl, Italy). Total nitrogen (N) was determined in feed, feces and urine by the Kjeldahl method (AOAC, 2001; method 2001.11) and crude protein (CP) was calculated as N × 6.25. Crude fat was determined by an

accelerated solvent extractor (ASE 200; Dionexm, Sunnyvale, CA, USA). The neutral detergent fiber fraction (NDF) was determined by Ankom-220 fiber analyzer (Ankom technology corp. Fairport, NY, USA) with heat-stable α-amylase and sodium sulfite (Van Soest et al., 1991), then expressed inclusive of residual ash. Starch in concentrate of the feed was analyzed according to standard procedures (AOAC, 1997; method 996.11). All the samples were first washed with 80% ethanol followed by α-amylase and heated in 100 °C water for 6 min. Then sodium acetate and amyl-glucosidase were added to the reaction tubes, vortex-mixed and heated in 50 °C water bath for 30 min. The reaction tubes were centrifuged for 10 min at 3000 × g, at room temperature, and samples were analyzed on a Roche Cobas Miras (Roche Diagnostics, Basel, Switzerland). The water-soluble carbohydrates of the silage samples were analyzed according to McDonald and Henderson (1964).

Milk Composition

The content of fat, protein, lactose, urea and free fatty acid (FFA) in the milk was analyzed by fourier-transform infrared (FTIR) spectroscopy analyzer (MilkoScan^{TM} Combifoss 6500; Foss Electric. A/S, Nils Foss Allé 1, DK-3400 Hillerød, Denmark).

Gas Samples

The concentration of methane and sulfur hexafluoride in samples of breath and ambient air (background) near the cow pen were determined by a gas chromatograph (Model 7890A, Agilent,

Santa Clara, CA, USA) equipped with a flame ionization detector for the analysis of methane, and an electron capture detector for the analysis of sulfur hexafluoride, by a 20-m wide-bore Poraplot Q (0.53 mm) column at 38 °C with back flushing. The flow rate of the carrier gas was 30 mL/min of N_2 for sulfur hexafluoride and 40 mL/min of helium for methane.

Rumen Fluid Samples

The determination of short-chain fatty acids in the rumen samples was according to Chen et al. (2020a). The determination of ammonia in the rumen samples was according to rapid ammonia assay kit (K-AMIAR, Megazyme Ltd., USA).

Calculations

Total tract apparent digestibility was calculated as (dry matter intake (g/d) – dry matter in fecal loss (g/d)) / dry matter intake (g) for each day of sampling separately. Organic matter (OM) was calculated as DM minus total ash.

Energy-corrected milk (ECM) yield was calculated as follows: ECM (kg) = milk (kg) × [(38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 16.54 × lactose (g/kg) + 20.7) /1340] (Sjaunja et al., 1991).

Daily enteric methane emission was calculated according to Lassey (2013):

$$\mathrm{CH_4(g/d)} = \frac{\mathrm{SF_6\ (g/d)} \times (\mathrm{[CH_4]_a} - \mathrm{[CH_4]_b})}{(\mathrm{[SF_6]_a} - \mathrm{[SF_6]_b}) \times (\mathrm{M_{CH_4}/M_{SF_6}})}$$

Where SF_6 is the predetermined release rate of sulfur hexafluoride from the permeation tube; $[CH_4]_a$ and $[SF_6]_a$ is the

concentration of methane and sulfur hexafluoride in the gas samples, respectively; $[CH_4]_b$ and $[SF_6]_b$ is the concentrations of methane and sulfur hexafluoride in the background ambient samples, respectively; M_{CH_4} and M_{SF_6} is the molecular weight of methane and sulfur hexafluoride, respectively.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS. The model included the fixed effect of pretreatment (T159 or control), treatment (T159 or control), the interaction of pretreatment and treatment, day [2 days: Table 3 (ECM and milk composition), Table 4 (CH_4 g/kg ECM), Table 6, Table S1; 3 days: Table 5 (digestibility); 5 days: Table 3 (DMI, milk yield), Table 4 (all CH_4 variables except CH_4 g/kg ECM) and Table 5 (intake)], and hour (Table 6). Cow was modeled as a random effect. The restricted maximum likelihood method was used to estimate the variance components (cow and residual). In addition, Kenward-Roger's option was used in the model statement to estimate denominator degrees of freedom. All the experimental data are presented as least square means ± standard error of the means and the statistical significance was declared at $P < 0.05$ and trends are discussed at $P < 0.10$.

RESULTS

Feed Intake, Milk Yield and Composition

The average DMI was 17.5 and 18.7 kg/day in exp1 and exp2, respectively (Table 3). Dry matter intake did not differ ($P > 0.10$) between cows administered with living and dead T159 in exp2, but the DMI in exp1 tended ($P < 0.10$) to be 1.1% lower in the T159 group compared to control (Table 3). The pretreatment (PT) effect was observed in the milk yield and DMI in exp1 ($P < 0.05$), and milk yield in exp2 ($P < 0.05$). The average ECM was 25.4 and 31.6 kg/day in exp1 and exp2. Supplying cows with living T159 had no effect ($P > 0.10$) on ECM, milk production efficiency (ECM/BW) and feed conversion efficiency (ECM/DMI) in exp1 and exp2 (Table 3). The contents of fat, protein, lactose, urea and FFA in milk did not differ ($P > 0.10$) between treatments (T) in exp1 and exp2, except for a slight increase ($P < 0.05$) of lactose and urea contents in milk of ruminally cannulated cows supplied with living T159 in exp2 (Table 3). Effects of PT, PT × T interactions and day were not observed ($P > 0.10$) except for an effect of day ($P < 0.05$) on milk urea and FFA in exp1, and milk yield (kg/day) in exp2. In addition, there was a PT × T interaction for milk lactose percentage in exp2 ($P < 0.05$).

Methane Production and Methane Emission Intensity

The average methane production was 383 g/day from intact cows of exp1 and 430 g/day from cannulated cows of exp2 (Table 4). Treatment and day had no effect ($P > 0.10$) on methane production (g/day), methane yield (g/kg DMI) and methane emission intensity (g /kg ECM, g/100 kg BW) in exp1 and exp2 (Table 4). Pretreatment and PT × T interactions were observed

only in exp1, with a pretreatment effect on methane emission intensity (g/kg ECM) and a PT × T interaction for nearly all methane variables ($P < 0.05$ for g/d and g/100 kg BW; $P < 0.10$ for g/kg DMI and g/kg ECM) except methane emission intensity (g/kg milk yield). In exp2, methane was also scaled to kg digested OM and digested NDF, and no effect ($P > 0.10$) of T, D, PT and PT × T was observed for these traits (Table 4).

Nutrient Intake and Digestibility

In exp2, nutrient intake and digestibility were determined (Table 5). Crude protein intake (CPI) did not differ ($P > 0.10$) between treatments, but the intake of OM and NDF was slightly higher ($P < 0.05$) and that of starch slightly lower ($P < 0.05$) in cows supplied with living T159. The CP intake tended to be influenced by PT ($P < 0.10$). Besides, we observed that all the parameters of nutrient intake were influenced by PT × T ($P < 0.05$), whereas day had no effect ($P > 0.10$). The apparent digestibility of OM and NDF was on average 76.3% and 66.8%, respectively. Treatment, D, PT and PT × T had no effect on the digestibility of OM and NDF, except for a trend ($P > 0.10$) of day affecting the digestibility of NDF.

Rumen Fermentation Parameters

In exp2, the rumen fermentation parameters were determined (Table 6). Treatment had no effect ($P > 0.10$) on molar concentration of total SCFAs, molar proportions of acetate (A) and propionate (P), the ratios of A : P and (acetate + butyrate) : propionate, molar concentration of ammonia and ruminal pH, but

388 the molar proportion of butyrate in total SCFAs was slightly
389 increased ($P < 0.05$) in rumen fluid of cows administered with
390 living T159. Pretreatment effects were observed ($P < 0.05$) for the
391 ratios of acetate : propionate and (acetate + butyrate) : propionate.
392 Moreover, PT × T interactions were observed for molar
393 concentration of total SCFAs ($P < 0.05$), molar proportion of
394 acetate ($P < 0.10$) and butyrate ($P < 0.05$) and ruminal pH ($P <$
395 0.05). All rumen fermentation variables were affected ($P < 0.05$)
396 by time (hour) of sampling in each sampling day. The day of
397 sampling had an effect ($P < 0.05$) on the proportions of ruminal
398 propionate and butyrate, the ratios of acetate : propionate and
399 (acetate + butyrate) : propionate, and ruminal pH.
400 ***Nitrogen Balance***
401 Treatment had no effect ($P > 0.10$) on absolute nitrogen (N)
402 intake, and on absolute (g/d) and relative (% of nitrogen intake)
403 milk nitrogen and body nitrogen retention (exp, Table S1). In
404 cows administered with living T159 an increase in the absolute
405 fecal nitrogen excretion ($P < 0.05$) and the fecal nitrogen
406 proportion of total nitrogen ingested ($P < 0.10$) was observed.
407 This was accompanied by a decline ($P < 0.10$) in the absolute
408 urinary nitrogen excretion and the urinary nitrogen proportion of
409 either total nitrogen ingested, or total nitrogen excreted. As a
410 consequence of the increase in fecal nitrogen losses, the apparent
411 nitrogen digestibility decreased ($P < 0.10$) in cows supplied with
412 living T159. Pretreatment and PT × T had no effect on variable of
413 nitrogen balance, except for a pretreatment effect for urinary

nitrogen proportion of total nitrogen excreted. In addition, we found an effect of day on daily nitrogen intake ($P < 0.05$) and body nitrogen retention (g/day) ($P < 0.10$).

DISCUSSION

The aim of this study was to determine the efficacy of *Propionibacterium thoenii* T159 on enteric methane emissions and performance of Norwegian Red cow at high feed intake *in vivo*. We hypothesized that the administration of living strain culture of T159 could mitigate the enteric methane emissions and increase the performance of Norwegian Red cow at high feed intake. However, living strain culture of T159 had no effect on enteric methane emissions and did not improve the production performance of Norwegian Red cow.

Propionibacteria and Methane Production

We measured the enteric methane emission by sulphur hexafluoride tracer technique in this study and ruminally cannulated cows were used in exp2. Therefore, the fermentation gas may escape from the rumen through the cannula of the cows without being collected and quantified. On the one hand, it is assumed that a potential leakage was proportional to the amount of gas in both treatments of exp2 and therefore it should allow comparison within one experiment. On the other hand, the higher DMI of the cows in exp2 compared to exp1 also led to a higher enteric methane emission and therefore we assume that the

methane quantification by sulphur hexafluoride tracer technique in this study was representative.

In exp2, the measurements of enteric methane emission were performed for five days (day 17, 18, 19, 20 and 21) and the samples of rumen content were collected for the other two days (day 14 and day 16; 13 time points in each day) in each period, thus the sampling of rumen contents via rumen cannula did not interfere with the quantification of enteric methane emission.

The substantial methane reduction in the propionibacteria treatment group (20% lower than the control group) we observed in the batch culture *in vitro* (Chen et al. 2020a) by the addition of T159 was inconsistent with the results in exp1 and exp2 *in vivo*, where no difference between treatments were observed in both experiments *in vivo*. In the study *in vitro* by Chen et al. (2020a), the control group was not added by dead strain culture of T159 but the basal diet with sodium lactate broth (SLB medium, the growth medium for propionibacteria). However, it is unlikely that the difference in control groups between our previous study *in vitro* (Chen et al. 2020a) and our present study *in vivo* explains the difference in the efficacy of T159 to reduce methane emission. More likely, the potentially more competitive conditions among microbiota *in vivo*, and the absorption and secretion processes in the rumen could explain the differences in results. In this study, the control cows received the dead strain culture of T159 (autoclaved bacteria) to eliminate the additional effects of microbial fermentation products (e.g. microbial nitrogen), and

nutrients, vitamins and/or minerals in the bacterial culture (Meale et al., 2014).We expected the dead strain culture to differ from living strain culture of T159 in the effect on enteric methane emission and performance. However, this was not the case. In the previous study *in vitro*, Meale et al. (2014) reported a lower ($P < 0.05$) methane emission in the treatment fermenter added living strain culture of T54 compared with the control fermenter added the dead strain culture (autoclaved *P. freudenreichii* T54) in the rusitec apparatus for 20 days, which are consistent with the study of Alazzeh et al. (2013) where only sterilized SLB medium was added in the control. These two reports indicated that the dead strain culture of propionibacteria seems had little impact on the rumen fermentation *in vitro*. This study is the first trial *in vivo* supplying the dead strain culture as the control and reporting the enteric methane emissions of cattle, whereas in other studies *in vivo* only basal diet or the carrier/SLB medium was offered in the control cows without any dead strain culture (Table 7). However, the type of the control group (dead strain culture in SLB medium, maltodextrin/lactose carrier only, no supplementation) seems not to be the reason for a lack in effect on enteric methane emissions *in vivo*, since none of the studies (summarized in Table 7) showed a decline in methane emissions regardless of the type of control used. The lack of effect on the mitigation of methane emission was observed in both gas sampling means including respiration chamber technique and sulfur hexafluoride tracer technique (in

this study) consistently, suggesting the type of gas sampling means had no effect either (Table 7).

The dose of propionibacteria offered to the dairy cows (8.5×10^{11} CFU/head × day) in this study *in vivo* was higher than in other studies reporting enteric methane emission from ruminants, including Vyas et al. (2016) who offered T159 to beef heifers and the studies which offered other strains of propionibacteria as summarized in Table 7. The dose of the strain T159 (1×10^{11} CFU/head × day) by Vyas et al. (2016) was lower than the dose of this study. Both trials (Vyas et al., 2016; this study) did not result in a decline in methane yield (g/DMI) or methane emission intensity (g/ECM; g/100 kg BW), which is consistent with other reports with a lower dose (Table 7). In summary, it is possible that the administration of a higher dose might not mitigate enteric methane emission of ruminants *in vivo*. Finally, the decisive factor might be the physiological condition or metabolic activity of exogenous propionibacteria rather than the dose of the strain offered to cattle. In our previous study, we reported that T159 might survive or stimulate other indigenous propionibacteria in the rumen for at least five days *in vivo* (Chen et al., 2020b). The pretreatment effect ($P < 0.05$) for methane emission intensity (g /kg ECM) (Table 4) in exp1 may indicate that T159 could survive or stimulate other indigenous propionibacteria even longer than five days in the rumen of intact cows.

In this study *in vivo*, the administration period was only three weeks in this study (Table 1) which is the same as Vyas et al.

516 (2016). In previous studies with methane emission results, the
517 administration periods were between 21 days and 28 days in cattle
518 (Table 7) but in sheep, the period was 70 days (Raphelis-Soissan
519 et al., 2014). None of these studies reported the mitigation of
520 enteric methane emission by propionibacteria significantly.
521 However, in the long-term (32 weeks) productive experiments of
522 dairy cattle by Stein et al. (2006) and Lehloenya et al. (2008b)
523 which did not report the enteric methane emission by the
524 administration of the strain P169, the improvement of milk
525 production ($P < 0.05$) was reported. Until now, it is still unknown
526 that whether the administration of propionibacteria in a long-term
527 (longer than 32 weeks) could mitigate the methane emission in
528 ruminants *in vivo*.
529 In line with other studies (Table 7), we found little difference
530 in DMI and methane emission between treatment groups either.
531 Nevertheless, we observed a pretreatment effect on DMI in both
532 experiments (exp1: $P < 0.05$, exp2: $P < 0.10$), which indicated a
533 carryover effect of propionibacteria administration to the rumen
534 fermentation pattern, or neuropeptide Y/appetite of cattle from the
535 previous period. In this study, cows presented a high DMI of
536 about 18 kg/head × day (Table 3), similar to Philippeau et al.
537 (2016). When the cows were at a high feeding level, the substrate
538 in the rumen could be abundant for the growth, metabolism and
539 reproduction of rumen microbiota. On the other side, the feed
540 passage time might be shorter, favoring only fast-growing
541 bacteria but not propionibacteria with a prolonged lag phase of 6

h in growth. Therefore, a higher feed intake level might not improve the effect of propionibacteria on the mitigation of enteric methane emission compared to the studies with lower feed intake levels (Vyas et al. 2014a,b, 2016; Jeyanathan et al. 2019). However, Lehloenya et al. (2008a) pointed out that feeding *P. jensenii* P169 to beef cattle did not affect particulate passage rate, liquid dilution rate, or turnover time in steer. It is still unknown that whether the strain T159 could affect particulate passage rate in the rumen of dairy cow and it should be investigated further.

Among the previous trials which focused on the enteric methane emission in cattle (Table 7), the forage to concentrate ratio (60 : 40) of the diet in this study was coincide with Vyas et al. (2016), and similar to Jeyanathan et al. (2019), Philippeau et al. (2016) and Vyas et al. (2014a), but was quite different from the corn grain finishing diet in the study of Vyas et al. (2014b). Until now, all the studies, including this study, did not indicate that the changing of the forage : concentration ratio in the diet had any effect on the mitigation of enteric methane emission by the administration of propionibacteria in cattle *in vivo*.

Consistent with previous reports with the administration of strains T159 and P63 *in vivo* (Vyas et al., 2014a,b; Philippeau et al., 2016), the administration of T159 in this study did not improve the digestibility of OM and NDF. Feed digestibility and enteric methane emission are positively related. The fact that the molar concentration of total SCFA and molar proportions of propionate and acetate in rumen content were not affected by

568 treatment is also in agreement with most previous reports which
569 focused on the effect of propionibacteria on methane emission
570 (Table 7). Especially, Vyas et al. (2016) found a lower ruminal
571 acetate proportion ($P < 0.05$) in the beef cattle offered T159.
572 Although the results in this study was slightly differed from Vyas
573 et al. (2016) possibly attributed to a higher dose of T159 and an
574 initial double dose (one Liter living or dead strain culture) on the
575 first day of each period, these differences might not explain the
576 difference in acetate response between the two studies. Although
577 we observed no treatment effect of any rumen fermentation
578 parameters except for an increase in butyrate proportion ($P <$
579 0.05) in the T159 treatment group, the effect of pretreatment and
580 pretreatment × treatment interactions were observed for several
581 parameters, indicating the carryover effect of the T159 treatment
582 to the next period of the experiment. In summary, the results of
583 this study *in vivo* were inconsistent with our promising findings
584 of elevated molar concentration of total SCFA, and molar
585 proportions of propionate reported in the study of Chen et al.
586 (2020a) *in vitro*. It is often observed that *in vitro* and *in vivo*
587 results are not in agreement. This is because the rumen digestion
588 processes *in vivo* including the interactions of microbials, the
589 absorption of SCFA, ammonia and other rumen fermentation
590 production, the urea cycle, neuromodulation, hormone regulation
591 and the endogenous secretions would be much more complicated
592 than the rumen simulation process *in vitro*.
593 ***Propionibacteria and Milk Production***

In this study, we offered dead strain culture (autoclaved strain T159 in SLB medium) to control cows, whereas none of the previous reports offered dead strain culture to control cows *in vivo*. However, we cannot evaluate the effect of the dead strain culture *in vivo* since we did not include a third treatment group in which the cows were received SLB medium only. The dead strain culture may contain several prebiotics, such as growing factors or allelochemicals which may stimulate the growth of specific microbials in the rumen and affect the milk production in some approaches. Akay and Dado (2001) reported that compared with the control group added only substrate, the treatment group added both dead strain culture of propionibacteria and substrate had a slightly higher concentration of total VFA, acetate and propionate, and a slightly lower acetate : propionate ratio in the batch culture *in vitro*; however, no statistical analysis for data comparison in these two groups were provided. Akay and Dado (2001) also pointed out that the acetate and propionate concentrations only decreased by 5% in the treatment groups added autoclaved propionibacteria (dead strain culture) compared with the treatment groups added living strain culture (1×10^3, 1×10^6, and 1×10^9 CFU/g) of propionibacteria in the trials *in vitro*. However, we lack the results of the trial *in vivo* to evaluate the effect of the dead strain culture in the rumen of cattle.

On the other hand, only a few studies reported an increased milk production (4% fat-corrected milk, kg/day) in dairy cows receiving freeze-dried propionibacteria in the maltodextrin carrier

compared to the control cows receiving maltodextrin carrier only (Stein et al., 2006). Different from this study, the control cows in several previous reports were offered maltodextrin carriers (Vyas et al., 2014a,b; Lehloenya et al., 2008a,b; Stein et al., 2006), dextrose carriers (Weiss et al., 2008) or lactose carriers (Philippeau et al., 2016; Raeth- Knight et al., 2007). Most previous trials used specific carriers to carry the propionibacteria in the freeze-dried powder form (Stein et al., 2016; Azzaz et al., 2015; Francisco et al., 2002) which was different from the living strain culture in the SLB medium in this study. It is still unclear that whether these carriers or the SLB medium had any effect on milk production. However, it seems that the type of the PB strains (freeze-dried powder of the strains, thawed strain from the freeze form or living strain culture of the strain in this study) offered in the trials *in vivo* has little different effects on milk production. Boyd et al. (2011) used Bovamine®, which is a kind of commercial products containing the combination of *Lactobacillus acidophilus* NP51 and *P. freudenreichii* NP24 in the freeze-dried powder form, and reported a greater milk yield ($P < 0.05$), a greater ECM ($P < 0.05$) and a greater true protein yield ($P < 0.05$) in the Bovamine® treatment group in a 12-week trial. By contrast, Bovamine® showed no effect on the milk production by Thompson (2011) who used different ways of propionibacteria administration (top dressed on the diets, rumen infusion and post-rumen infusion) for cattle and found no different effects on milk production among these methods in a 5-week trial. It seems that a

longer administration period may lead to an improvement on the milk production of cattle regardless of the type of the strains (freeze-dried powder or living strain culture) offered to the cows, which could also explain the failure in the promotion of the milk yield in this study with a short period (3 weeks) administration. In the trial with a 3-week administration period, Thompson (2011) found no effect on the milk production by Bovamine®. Similarly, none of the four trials (Jeyanathan et al., 2019; Philippeau et al., 2017; Ferraretto et al., 2015; Morsy et al., 2014) with a short administration period (less than 6 weeks) showed a significant effect on the milk production. The long-term administration may have significant improvement (Stein et al., 2006) on milk production (4% fat-corrected milk, kg/day) but there were still opposite findings such as Weiss et al. (2008) and Reath-Knight et al. (2007). Moreover, it is still unknown that whether the dose of the strain has any effect on milk production. Most milk production reports, including this study, offered a similar dose (10^9 ~ 10^{11} CFU/head × day) but the effect on milk production were various. In summary, the dose effect of the propionibacteria strains needs to be investigated further.

According to the previous reports, it seems that there was little relationship between the milk production and DMI of the cattle by propionibacteria administration. Several studies *in vivo* reported an elevated ($P < 0.05$) DMI and milk yield (Azzaz et a., 2015; Ondarza et al., 2008) by the administration of P169 or the combination of P169 and yeast. Sawall et al. (2015) and Weiss et

al. (2008) reported a declined DMI by P169 (Sawall et al., 2015: $P < 0.10$; Weiss et al., 2008: $P < 0.05$) but no effect on milk production. By contrast, Francisco et al. (2002) showed a declined DMI ($P < 0.01$) and an elevated milk production ($P < 0.05$). Moreover, several reports (Boyd, et al., 2011; West et al., 2011) showed an elevated milk production ($P < 0.05$) with an insignificant difference of DMI by PB administration. Especially, West et al. (2011) reported an elevated feed conversion efficiency (ECM/DMI) ($P < 0.05$), which may indicate a higher feed efficiency by Bovamine®. Whereas, Arriola et al. (2011) and Boyd et al. (2011) did not found any effect in dairy efficiency (milk yield/DMI) by PB strains which may be attributed to the short administration period. Similarly, Jeyanathan et al. (2019) did not found any effect in feed conversion efficiency (ECM/DMI) by a 5-week administration of the strain 53W. Consistently, there was also no significant difference of feed conversion efficiency (ECM/DMI) between T159 treatment group and control group in this study. However, we observed a pretreatment effect of milk yield ($P < 0.05$) and DMI (exp1: $P < 0.05$; exp2: $P < 0.10$) in both experiments of this study which may indicate a carryover effect of the PB administration from the previous period to the subsequent period.

In this study, the strain T159 had no impact on milk production and the digestibility of organic matter in the 3-week administration trial. Consistent with this study, most other studies with similar short administration periods (Philippeau et al., 2017;

Morsy et al. 2014) showed no effect on both milk production and the digestibility. Nevertheless, all the previous reports with an improved milk production showed an elevated digestibility of the diet (Azzaz et al., 2015; Boyd et al., 2011), in which the administration periods are longer than 8 weeks. However, several long-term trials also reported no effect by PB administration. Reath-Knight et al. (2007) found no effect on both milk production and feed digestibility in a 12-week trial.

In this study, the strain T159 had no effect on milk production due to the little effect on ruminal fermentation parameters. Thompson (2011) and Reath-Knight et al. (2007) also reported no effect of propionibacteria administration on milk production and rumen fermentation parameters, consistent with this study. The percentage of lactose and the molar concentration of urea in milk was slightly increased by T159 administration in exp2, but not in exp1. Based on the studies reporting both results of SCFA and milk production, only Stein et al. (2006) reported an improved rumen fermentation parameters (SCFA, propionate and acetate : propionate ratio) resulting in an improved milk production (4% fat-corrected milk, kg/day, $P < 0.05$) in a 32-week trial by the administration of a slightly lower dose of *Propionibacterium jensenii* P169 (6×10^{11} CFU/head × day) than the dose of this study (8.5×10^{11} CFU/head × day). On the contrary, similar trials from Weiss et al. (2008) reported a lower total SCFA ($P < 0.05$), a greater propionate ($P < 0.05$), a lower acetate : propionate ratio ($P < 0.10$) by *Propionibacterium*

jensenii P169 but no effect on milk production in a 19-week trial. Propionibacteria may produce propionate in the rumen and improve the milk production in a few cases. However, the relationship between the manipulation of rumen fermentation pattern and the improvement of milk production by propionibacteria is still unclear. Further investigation with the long-term trial may be necessary for the elucidation of the mechanism.

As far as we know, this study is the first report on the nitrogen balance of dairy cows by PB administration (Table S1). The results showed that the strain T159 increased nitrogen excretion in the feces ($P < 0.05$) but decreased the nitrogen excretion in the urine ($P < 0.05$). The increased fecal nitrogen excretion agrees with the observed by tendency lower nitrogen digestibility in this study. Consistently, Ebeid et al. (2013) reported the nitrogen balance of sheep fed with Dairy ProP169® (1.2×10^{10} CFU/head × day) and showed a slightly higher nitrogen in the feces ($P > 0.10$) and a slight lower nitrogen in the urine ($P > 0.10$) but the reason was unclear. In contrast to this study, Lehloenya et al. (2008a) showed the combination of *P. jensenii* strain P169 and yeast had no impact on the nitrogen intake, fecal nitrogen and nitrogen digestibility in steers. Moreover, Lehloenya et al. (2008b) reported that the combination of *P. jensenii* strain P169 and yeast had no effect on urinary nitrogen excretion (g/day) in dairy cows and pointed out that the levels of urinary nitrogen excretion and milk urea nitrogen reflect

differences in body growth, milk production and DMI. Similar to exp1, but in contrast to exp2 of our study, Lehloenya et al. (2008b) also reported that the combination of *P. jensenii* strain P169 and yeast had no effect on milk urea nitrogen (mg/dL) in both multiparous and primiparous Holstein dairy cows. Stein et al. (2006) showed a greater concentration of urea nitrogen (MUN) in the milk of Holstein dairy cows supplied with *P. jensenii* strain P169 from week 13 to week 25 of the trial. In this study, we observed a higher MUN in exp2 ($P < 0.05$) but there was no difference of MUN in exp1. Also, Ferraretto et al. (2015), Weiss et al. (2008), Raeth-Knight et al. (2007) and Francisco et al. (2002) found no effect of PB strains on MUN. The elevation of MUN may be attributed to the elevated ammonia incorporation into microbial protein in the rumen (Stein et al., 2006). The increased microbial protein may improve the protein supply for synthesis of milk protein and decrease the loss of nitrogen in dairy cattle (Stein et al., 2006). On the other side, milk urea nitrogen or milk urea contents could be regarded as a reflection of the concentration of blood urea nitrogen (BUN) (Lehloenya et al., 2008b). Thus, milk urea nitrogen or milk urea contents might be considered as an index of inefficient nitrogen utilization in lactating dairy cows (Lehloenya et al., 2008b). Higher milk urea contents in exp2 of this study might be unfavorable according to Lehloenya et al. (2008b), which might be attributed to the rumen cannula of the cows in exp2. Further investigation could be

necessary for the exploration of the nitrogen balance in lactating Norwegian Red cows.

CONCLUSION

In conclusion, in our two short-term experiments, the strain T159 demonstrated limited ability to improve the ruminal propionate production and milk production, and failed to mitigate enteric methane emissions. Besides, although T159 could slightly promote the intake of OM and NDF, the nutrition digestibility of dairy cows was not improved and their effect on the nitrogen balance was unfavorable. These results may be related to the fact that strain T159 cannot integrate, persist and compete with the rumen microbiota, or may also be related to the incapability of T159 to maintain high metabolic activity in the rumen in a short-period administration.

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Table 1. Experimental design of experiment 1 (intact cows) and experiment 2 (cannulated cows)

Period	Animals				
Experiment 1[1]	Cow 1 (La[3] 2)	Cow 2 (La 2)	Cow 3 (La 2)	Cow 4 (La 2)	Cow 5 (La 2)
I	C[4]	T159	C	C	T159
II	T159[5]	C	T159	T159	C
III	T159	C	C	T159	C
IV	C	T159	T159	T159	C
Experiment 2[2]	Cow 6 (La 5)	Cow 7 (La 4)	Cow 8 (La 3)	Cow 9 (La 2)	
I	C	T159	T159	C	
II	C	T159	C	T159	
III	T159	C	C	T159	

[1]Experiment 1 = 16 d adaptation phase + 5 d sampling phase = 21 d of each period.
[2]Experiment 2 = 12 d adaptation phase + 9 d sampling phase = 21 d of each period.
[3]La = Number of lactations.
[4]C = Control group, the cows received dead strain culture of *Propionibacterium thoenii* T159 by the autoclave (121.30 °C, 30 min) of the living strain culture of T159.
[5]T159 = Treatment group, the cows received living strain culture of *Propionibacterium thoenii* T159 grown in the SLB medium at 30 °C for 48 h.

Table 2. Ingredients and chemical composition of the basal diet in experiment 1 (intact cows) and experiment 2 (cannulated cows)

Item	
Ingredient, % of DM	
Silage	60.0
Timothy (*Phleum pretense*)	30.0
Common meadow grass (*Poa pratensis*)	12.0
Meadow fescue (*Festuca pratensis*)	12.0
White clover (*Trifolium repens*)	6.00
Concentrate	40.0
Barley (*Hordeum vulgare*)	17.8
Rapeseed meal (*Brassica napus*)	6.10
Soybean meal (*Glycine max*)	4.36
Wheat bran (*Triticum aestivum*)	2.57
Sugarcane molasse (*Saccharum officinarum*)	2.42
Wheat (*Triticum aestivum*)	2.38
Vegetable oil and fat (Akofeed nøt, AAK AB, Malmö)	1.19
Mineral-vitamin mixture	1.19
Corn gluten (*Zea mays*)	0.792
Limestone	0.488
Pea residue (*Pisum sativum*)	0.396
Soybean oil (*Glycine max*)	0.316
Chemical composition, % of DM	
Silage	
Organic matter	93.4
Crude protein	11.0
Neutral detergent fiber	63.3
Crude fat	2.84
Water-soluble carbohydrates	11.0
Concentrate	
Organic matter	92.4
Crude protein	22.1
Neutral detergent fiber	16.5
Crude fat	5.49
Starch	33.3

Table 3. Feed intake, milk yield and milk composition from lactating cows administered with *Propionibacterium thoenii* T159 in experiment 1 (intact cows) and experiment 2 (cannulated cows)

	Treatment			*P*-value			
Item	Control[1]	T159[2]	SEM	T[3]	D[4]	PT[5]	PT × T
Experiment 1 (intact cows)							
DMI, kg/d	17.6	17.4	0.064	0.054	0.55	0.009	0.25
Milk, kg/d	25.3	24.5	0.356	0.43	0.68	0.017	0.33
ECM, kg/d	25.6	25.2	0.495	0.76	0.33	0.21	0.28
ECM per BW, kg/100 kg	4.38	4.32	0.105	0.70	0.34	0.24	0.34
ECM per DMI, kg/kg	1.46	1.43	0.027	0.67	0.45	0.62	0.35
Milk composition							
Fat, %	3.90	3.95	0.077	0.68	0.14	0.75	0.67
Protein, %	3.13	3.26	0.084	0.25	0.45	0.48	0.24
Lactose, %	4.67	4.68	0.030	0.65	0.79	0.79	0.80
Urea, mmol/L	3.75	3.72	0.128	0.86	0.033	0.31	0.85
FFA, mol/dL	0.880	0.770	0.076	0.43	0.049	0.70	0.30
Experiment 2 (cannulated cows)							
DMI	18.8	18.6	0.107	0.69	0.99	0.066	0.58
Milk, kg/d	29.1	28.9	0.762	0.64	0.007	0.022	0.53
ECM, kg/d	30.7	32.5	1.012	0.15	0.76	0.22	0.28
ECM per BW, kg/100 kg	4.97	5.03	0.12	0.33	0.71	0.15	0.45
ECM per DMI, kg/kg	1.64	1.75	0.054	0.16	0.72	0.20	0.37
Milk composition							
Fat, %	4.06	4.38	0.110	0.10	0.67	0.41	0.28
Protein, %	3.05	3.17	0.045	0.23	0.97	0.45	0.67
Lactose, %	4.72	4.81	0.018	0.002	0.19	0.18	0.043
Urea, mmol/L	3.12	3.49	0.106	0.015	0.17	0.42	0.20
FFA, mol/dL	0.575	0.775	0.112	0.47	0.89	0.99	0.92

[1]Control = The cows received dead strain culture of *Propionibacterium thoenii* T159 by autoclaving (121.30 °C, 30 min) the living strain culture of T159.
[2]T159 = The cows received living strain culture of *Propionibacterium thoenii* T159 grown in the SLB medium at 30 °C for 48 h.
[3]T = Treatment; [4]D = Day (days 17, 18, 19, 20 and 21 for milk yield and DMI; day 19 and day 21 for other parameters in the table); [5]PT = Pretreatment.

Table 4. Methane production, yield and emission intensity from cows administered with dead (control) or living (T159) *Propionibacterium thoenii* T159 in experiment 1 (intact cows) and experiment 2 (cannulated cows)

	Treatment			P-value			
Item	Control[1]	T159[2]	SEM	T[3]	D[4]	PT[5]	PT × T
Experiment 1 (intact cows)							
g/d	381	385	9.62	0.55	0.40	0.29	0.049
g/kg DMI	21.7	22.0	0.558	0.70	0.34	0.25	0.079
g/kg milk yield	15.8	15.8	0.476	0.54	0.41	0.20	0.12
g/kg ECM	15.3	15.3	0.440	0.56	0.50	0.042	0.057
g/100 kg BW	65.1	65.8	0.656	0.49	0.39	0.30	0.022
Experiment 2 (cannulated cows)							
g/d	440	419	15.2	0.92	0.53	0.56	0.34
g/kg DMI	23.3	22.5	0.873	0.79	0.54	0.51	0.33
g/kg dOMI[6]	24.3	24.0	0.928	0.79	0.54	0.51	0.33
g/kg dNDFI[7]	63.6	60.6	2.26	0.58	0.53	0.79	0.33
g/kg milk yield	16.0	15.1	0.835	0.24	<0.001	0.79	0.75
g/kg ECM	13.7	13.6	0.524	0.71	0.66	0.59	0.99
g/100 kg BW	67.6	67.7	2.53	0.60	0.61	0.34	0.90

[1]Control = The cows received dead strain culture of *Propionibacterium thoenii* T159 by autoclaving (121.30 °C, 30 min) the living strain culture of T159.
[2]T159 = The cows received living strain culture of *Propionibacterium thoenii* T159 grown in the SLB medium at 30 °C for 48 h.
[3]T = Treatment.
[4]D = Day (days 17, 18, 19, 20 and 21 for milk yield and DMI; day 19 and day 21 for other parameters in the table).
[5]PT = Pretreatment.
[6]dOMI = Digestible organic matter intake.
[7]dNDFI = Digestible neutral detergent fiber intake.

Table 5. Nutrient intake and digestibility variables from lactating cows administered with dead (control) or living (T159) *Propionibacterium thoenii* T159 in experiment 2 (cannulated cows)

	Treatment			*P*-value			
Item	Control[1]	T159[2]	SEM	T[3]	D[4]	PT[5]	PT × T
Daily intake, kg/day							
OM[6]	17.5	17.6	0.102	0.001	0.69	0.10	0.001
NDF[7]	6.80	6.90	0.019	<0.001	0.72	0.60	0.018
CP	2.70	2.68	0.004	0.198	0.88	0.050	0.001
Starch	2.48	2.38	0.106	<0.001	1.00	0.57	0.005
Apparent digestibility, %							
OMD[8]	76.2	76.3	0.526	0.99	0.22	0.76	0.70
NDFD[9]	67.3	66.2	0.780	0.43	0.091	0.28	0.63

[1]Control = The cows received dead strain culture of *Propionibacterium thoenii* T159 by autoclaving (121.30 °C, 30 min) the living strain culture of T159.
[2]T159 = The cows received living strain culture of *Propionibacterium thoenii* T159 grown in the SLB medium at 30 °C for 48 h.
[3]T = Treatment.
[4]D = Day (days 17, 18, 19, 20 and 21 for intake; days 19, 20 and 21 for digestibility).
[5]PT = Pretreatment.
[6]OM = Organic matter.
[7]NDF = Neutral detergent fiber.
[8]OMD = The apparent digestibility of organic matter.
[9]NDFD = The apparent digestibility of neutral detergent fiber.

Table 6. Rumen fermentation parameters from lactating cows administered with dead (control) or living (T159) *Propionibacterium thoenii* strain T159 in experiment 2 (cannulated cows)

	Treatment			*P*-value				
Item	Control[1]	T159[2]	SEM	T[3]	D[4]	H[5]	PT[6]	PT × T
SCFAs, mmol/L	91.9	99.4	1.47	0.14	0.11	<0.001	0.58	0.033
mol/100 mol								
Acetate (A)	67.4	72.1	1.19	0.20	0.60	<0.001	0.89	0.081
Propionate (P)	14.7	15.7	0.274	0.83	<0.001	<0.001	0.11	0.18
Butyrate (B)	9.63	9.73	0.131	<0.001	<0.001	<0.001	0.20	<0.001
A : P ratio	4.82	4.80	0.072	0.39	<0.001	<0.001	0.044	0.49
(A + B) : P ratio	5.50	5.44	0.076	0.28	<0.001	<0.001	0.045	0.66
Ammonia, mM	22.9	23.1	1.28	0.24	0.52	<0.001	0.11	0.45
pH	6.52	6.45	0.020	0.57	0.001	<0.001	0.89	0.003

[1]Control = The cows received dead strain culture of *Propionibacterium thoenii* T159 by autoclaving (121.30 °C, 30 min) the living strain culture of T159.
[2]T159 = The cows received living strain culture of *Propionibacterium thoenii* T159 grown in the SLB medium at 30 °C for 48 h.
[3]T = Treatment.
[4]D = Day (day 14 and day 16).
[5]H = Hour (07.00, 07.30, 08.00, 09.00, 10.00, 12.00, 14.00, 14.30, 15.00, 16.00, 17.00, 19.00 and 21.00).
[6]PT = Pretreatment.

999 **Table 7. Overview of studies reporting the effect of propionibacteria on enteric methane emission from ruminants**

Publication	Animal type	Strain	Dose, CFU/cow × day	The administration means of strains for cattle	Duration of administration (day)	Decline in methane yield or emission intensity	Increase propionate proportion (mol/100 mol SCFA)
Raphelis-Soissan et al., 2014	Sheep	*P. acidipropionici*	1.15×10^{11}	Diluted in water and sprayed on 10 g wheat bran (diet). Control sheep received only basal diets.	70	No	No
Jeyanathan et al., 2016	Sheep	*P. freudenreichii* 53W	6×10^{10}	The frozen form of strain was thawed in 0.1% sterile peptone solution for administration. Control sheep received only basal diets.	28	No	No record
Vyas et al., 2014a	Beef Heifer (BH)	*P. acidipropionici* P169; *P. acidipropionici* P5; *P. jensenii* P54	5×10^{9}	In 10 g of a maltodextrin carrier using a porcine gel capsule. Control cows received the carrier.	28	No	No
Vyas et al., 2014b	BH	*P. acidipropionici* P169; *P. acidipropionici* P5; *P. jensenii* P54	5×10^{9}	The same as Vyas et al., 2014a	28	No	No
Vyas et al., 2016	BH	*P. freudenreichii* T114; *P. thoenii* T159; *P. freudenreichii* T54	1×10^{11}	The same as Vyas et al., 2014a	21	No	No
Philippeau et al., 2017	Holstein cow (HC)	*Propionibacterium* spp. P63	1×10^{10}	Offered in lactose carrier. Control cows received lactose carrier.	28	No	No
Jeyanathan et al., 2019	HC	*P. freudenreichii* 53-W	2.9×10^{10}			No	No

This study, exp1	Norwegian Red cow (NR)	*P. thoenii* T159	8.5×10^{11}	Living strain culture in SLB medium. Control cows received the same amount of dead strain culture.	21	No	No
This study, exp2	NR	*P. thoenii* T159	8.5×10^{11}	The same as exp1 in this study.	21	No	No

1000

APPENDIX

Supplementary Table S1. Nitrogen balance of the lactating cows administered with dead (control) or living (T159) *Propionibacterium thoenii* T159 in experiment 2 (cannulated cows)

	Treatment			*P*-value			
Item	Control[1]	T159[2]	SEM	T[3]	D[4]	PT[5]	PT × T
N[6], g/d							
Intake	428	427	3.63	0.99	0.023	0.84	0.99
Feces	116	133	4.12	0.039	0.31	0.20	0.92
Urine	134	118	3.95	0.060	1.00	0.11	0.36
Milk	155	148	5.42	0.43	0.96	0.15	0.60
Body	23.1	27.5	7.46	0.89	0.092	0.38	0.47
N balance, % of N intake							
Feces	27.2	31.1	0.978	0.050	0.14	0.19	0.90
Urine	31.2	27.7	0.891	0.070	0.48	0.13	0.34
Milk	36.2	34.7	1.19	0.36	0.41	0.18	0.54
Body	5.37	6.45	1.74	0.91	0.10	0.37	0.40
N utilization							
Apparent N digestibility, %	72.8	68.9	0.978	0.050	0.14	0.19	0.90
Urinary N, % of N excreted	53.4	47.2	1.19	0.005	0.34	0.031	0.61

[1]Control = The cows received dead strain culture of *Propionibacterium thoenii* T159 by autoclaving (121.30 °C, 30 min) the living strain culture of T159.
[2]T159 = The cows received living strain culture of *Propionibacterium thoenii* T159 grown in the SLB medium at 30 °C for 48 h.
[3]T = Treatment.
[4]D = Day (day 19 and day 21).
[5]PT = Pretreatment.
[6]N = Nitrogen.