ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(3): 513-521
doi: 10.25083/rbl/24.3/513.521

**Received for publication, December, 10, 2018**
**Accepted, January, 4, 2019**

*Original paper*

# *In vitro* effects of phenolic acids and IgY immunoglobulins on aspects of rumen fermentation

**MIHAELA BERCHEZ[1], ADRIANA CRISTINA URCAN[1], NICOLAE CORCIONIVOSCHI[2], ADRIANA CRISTE[1*]**

[1]Department of Microbiology and Immunology, University of Agricultural Sciences and Veterinary Medicine, Faculty of Animal Science and Biotechnologies, Cluj-Napoca, Romania
[2]Agri-Food and Biosciences Institute, Bacteriology Branch, Belfast, United Kingdom

**Abstract**

The current study was carried out in order to determine the effects of simple phenols and of specific anti-methanogen IgY antibodies on ruminal gas production, methane emissions, volatile fatty acids (VFA) profile and pH in in vitro ruminal cultures. Caffeic and p-Coumaric acids and IgY antibodies were added anaerobically to ruminal batch cultures. Ruminal parameters were measured after 24, 48 and 72 hours of incubation. The results showed that addition ofboth phenolic acids and IgY antibodies significantly ($P < 0.05$) decreased methane production at 24 and 48 hours of incubation. At 24 and 48 hours of incubation some significant differences were observed in volatile fatty acids profile, while the pH was not affected by simple phenols and IgY antibodies addition.

Simple phenols and IgY avian antibodies can be further tested in order to achieve the purpose of methane mitigation strategies, but the ideal way to inhibit the methanogenesis process in rumen would reduce methane production without altering the other ruminal parameters, such as VFAs, total gas production or pH.

**Keywords** Mitigation, phenols, antibodies, methane, methanogenesis

**To cite this article:** BERCHEZ M, URCAN AC, CORCIONIVOSCHI N, CRISTE A. Effects of phenolic acids and IgY immunoglobulins on aspects of rumen fermentation*, in vitro*. *Rom Biotechnol Lett*. 2019; 24(3): 513-521. DOI: 10.25083/rbl/24.3/513.521

*Corresponding author: ADRIANA CRISTE, Department of Microbiology and Immunology, University of Agricultural Sciences and Veterinary Medicine, Faculty of Animal Science and Biotechnologies, 3-5 Manastur Street, Cluj-Napoca 400372, Romania; E-mail:adriana.criste@usamvcluj.ro

## Introduction

Methane gas production from anthropogenic activities is of concern worldwide for its contributions to the accumulation of greenhouse gases. The agriculture sector is responsible for up to 18% of the total anthropogenic greenhouse gases emissions annually (STEINFELD & al. [1]) and livestock represents approximately 80% of these emissions (SEJIAN & al. [2]). Methane originates from enteric fermentation in animals, storage of manure, and anaerobic ecosystems.Damage to the air quality can also induce harmful effects on human health (an indirect impact) for the people living in the surroundingarea (RAHOVEANU & al [3]).

In livestock sector, enteric fermentation represents the main source of methane emissions in the atmosphere. Enteric fermentation is the process that results from a complex microbiological activity and affects mainly the ruminants (FORABOSCO & al. [4]).

The rumen represents a unique compartmentalized bioreactor and is characterized by a complex microbial community, predominantly obligate anaerobe micro-organisms (KUMAR & al. [5], LOZANO & al. [6]). The most abundant microorganisms of the rumen are bacteria with at least 50 bacterial genera (1010-1011 ml-1), followed by ciliate protozoa (106 ml-1) with 25 genera, 6 genera of fungi (106 ml-1), methanogenic Archaea (108-109 ml-1) and bacteriophages (LOZANO & al. [6]). These are the organisms that carry out the degradation of ingested plant materials into fermentation products (volatile fatty acids, H2) (JANSSEN [7]). Some of the fermentation products are absorbed across the rumen epithelium and are used as energy by ruminants (JANSSEN [7]).

Methanogens which represents the Archaea domain are characterized by the ability to scavenge H2 and CO2 produced by fermentative microorganisms of the ruminal microbiome (PATRA & al. [8]), producing methane under anoxic conditions (GUO et al. [9]). The major substrates used by methanogens to produce methane are CO2, compounds containing methyl group and acetate (LIU & WHITMAN [10]). Hydrogenotrophic pathway is the predominant way that uses CO2 as carbon source and H2 as electron donor (MORGAVI & al. [11]). Other substrates that are available for rumen methanogens are formic acid used by rumen hydrogenotrophic methanogens (HUNGATE & al. [12], ROTHER & KRZYCKI [13]) and methhyla-mines used by methylotrophic methanogens of the order *Methanosarcinale*s and the order *Methanobacteriales* (LIU & WHITMAN [10]). In rumen, methane is also produced via the aceticlastic pathway from acetate (LIU & WHITMAN [10]).

An interaction occurs between methanogens and the other rumen microbes, where H2 is transferred through species (PATRA & al. [8]). The association of hydrophobic methanogens with hydrogen producers' microorganisms is realized by attachment and by floc formation (THIELE & al. [14], LANGE & al. [15]). The symbiotic association of methanogens with ciliates is both intracellular and extracellular (SHARP & al. [16]), and generates up to 37% of rumen methane emissions (FINLAY & al. [17]).

Ruminal methane emission is of concern world-wide, because of its implication on the accumulation of greenhouse gases in the atmosphere (HOOK & al. [18]). Also, about 2-12% of gross energy intake produced by ruminal fermentation is converted to methane, which leads to the loss of feed energy for the animal (JOHNSON & JOHNSON [19]).

In ruminants, the major factors that affect methane gas production are volatile fatty acids, pH, the diet, animal species and stress (KUMAR et al. [5]). Diet is an important factor that has a high impact on methanogen numbers and can change the ruminal fermentation process (KUMAR & al. [5]). The profile of volatile fatty acids is affected by a number of factors, primarily the type and quality of feed (FRIGGENS & al. [20]), and reflects the quantity of methane production.

In the past decade, ruminal methanogens and all the topics in which they are included have attracted much research. The main aim is to understand their community structure, relationship with other microorganisms and diversity. Their study is particularly important in methane mitigation strategies, which can be effective in two ways. The first way represents a direct effect on methanogens, and the second way is the indirect effect caused by the impact on substrate availability. This strategy implies the effect on other ruminal microbes (HOOK & al. [18]).

Strategies that were studied in order to mitigate ruminal methane emissions include: chemical suppression (LEE & al. [21]), defaunation (HOOK & al. [18]) which represents the removal of ruminal protozoa from the rumen, uses of antibiotics such as monensin (BERGEN & BATES [22], RUSSEL & STROBEL [23]; GUAN & al. [24]), uses of organic acids (WOOD & al. [25]), of bacteriocins (RENUKA & al. [26]) such as nisin (MARTIN & al. [27]) and bovicin HC5 (LEE & al. [21]). Other approaches are the use of plant secondary metabolites and other plantcompounds such as tannins (CLARK [28]; CLARK & al. [29]; HESS & al. [30]), saponins (GOEL & al. [31]; BODAS & al. [32], HESS & al. [33]), essential oils (BEAUCHEMIN & McGIN, [34]), and another strategy is the development of a vaccine that can stimulate the ruminant immune system to produce antibodies against methanogens (WRIGHT & al. [35]).

Plant secondary metabolites are synthesized by plants and represent the group of chemicals that are not involved in the primary biochemical processes of plant growth (KAMRA & al. [36]). Several thousand of different plant secondary metabolites have been recorded (HARTMANN [37]) and some of them have shown antimicrobial activity (BODAS & al. [38], JAYANEGARA [39]).

COOK & al. [40], developed a novel adaptation of the use of vaccination that was described by WRIGHT & al. [35]. The research involved the passive immunization and the use of a non-invasive source of antibody (IgY) from chicken egg yolks. The results showed that specific anti-methanogen antibodies can be effective during the ruminal fermentation process.

The objective of this study was to determine the effects of two phenolic acids, caffeic acid and p-coumaric acid, and of specific anti-methanogen IgY antibodies on ruminal fermentation process *in vitro*.

## Materials and Methods

In this study, two sources of simple phenols (caffeic and p-coumaric acids) and specific anti-methanogens antibodies were evaluated for their effect on ruminal fermentation parameters.

### *1. Preparation of phenolic acids*

Caffeic acid and p-coumaric acid were purchased from Sigma Aldrich GmbH and prepared in sodium phosphate buffer (PBS) with pH 6.7 and stored at 4°C until use.

### *2. Preparation of avian IgY immunoglobulins*

*Preparation of methanogenic antigen and immunization*

Two strains of rumen methanogens were used to develop the methanogenic antigen. *Methanobrevibacter gottschalkii* (DSMZ 11977) and *Methanobrevibacter ruminantium* (DSMZ 1093) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkuturen (DSMZ) (Braunschweig, Germany). The antigen was prepared after the protocol of WRIGHT & al. 2004. Briefly, 0.2 ml formaldehyde 70% was added to 5 ml of methanogens culture. The cells were concentrated by centrifugation at 14,000 rpm for 10 min at 5°C, and then resuspended in sterile PBS. Supernatant was removed and the pellet was resuspended in sterile PBS, and this wash cycle was repeated three times. Freund's Complete Adjuvant was added to an equal volume of formalin-killed mixed cell solution. Protein concentration was determined by spectro-photometry (The Thermo Scientific NanodropTM 1000). Immunizations were conducted by injection into the pectoral muscle of laying hens in two rounds: day 1 and day 21.

*IgY isolation and quantification*

Hen eggs were collected and used for IgY isolation based on extraction with water and precipitation with ammonium sulfate method of CRISTE [41]. Briefly, the yolk is separated from albumen by washing with PBS buffer and passed on a filter paper. The vitelline membrane is than carefully removed. The yolk is 6-time initial yolk volume diluted with distilled water and mixed for 6 hours at 4°C. The lipid separation from water phase is performed by centrifugation at 10000g for 25 minutes at 4° C. IgY is obtained by fractionate precipitation with ammonium sulphate.

IgY immunoglobulins quantification was performed using a NanoDrop TM 1000 Spectrophotometer at 280 nm wave length.

### 3. *In vitro batch cultures incubation and fermentation*

Batch culture incubation was conducted to assess the effects of caffeic and p-coumaric acids and avian IgY antibodies on in vitro cumulative gas production, methane emissions, pH, and volatile fatty acids.

An adult rumen cannulated sheep was used as a donor for rumen fluid, which was collected before morning feeding. Ruminal fluid was filtered through sterile sieve, homogenized and kept in an anaerobic chamber. Forty milliliters of 141 DSM modified culture medium for Methanogens (Na2S x 9H2O was substituted with Cysteine-HCl x H2O and Na-acetate was eliminated from the medium preparation) and ten milliliters of ruminal fluid were dispensed anaerobically into 100 ml serum bottles containing 0.5 g of grass hay as substrate.

The phenolic acids were added in 12 mM acids 12 mM/ IgY immunoglobulins) along with controls (0.5 g of substrate, 10 ml rumen fluid and 40 ml medium).

Concentration shortly after dispensing the culture medium IgY antibodies were added in aliquots of 1 ml phenolic acids and IgYimmunoglobulins (0.5 g substrate, 10 ml rumen fluid, 40 ml culture medium and caffeic/p-coumaric.

### *4. Measurement of in vitro fermentation parameters*

At 24, 48 and 72 hours of incubation, the following ruminal parameters were analyzed: cumulative gas volume, methane production, pH and volatile fatty acids. Cumulative gas volume was measured using a water-displacement device (FEDORAK & HRUDEY [42]). A sample from the head space gas was transferred to a 20 ml GC vial for the methane analysis by gas chromato-graphy (Chrompack MicroGC CP-2002P) and the incubated

inoculums were sub sampled for pH and VFAs analysis by HPLC (SHIMADZU HPLC with HiPlex Agilent column and H2SO4 5 mM as eluent).

### *5. Statistical analysis*

All incubations were performed in triplicate. Significant differences between samples were analyzed with one-way ANOVA post hoc tests, and pairwise multiple comparisons were conducted using Tukey's test. Significant differences were reported based on $P < 0.05$. Statistical analyses were performed using the SPSS programme (SPSS Inc., Chicago, IL, USA).

## Results and Discussions

The effects of p-coumaric acid, caffeic acid and IgY avian-antibodies on cumulative gas volume and methane production are presented in Table 1. P-coumaric and caffeic acids were added to batch cultures in a 12 mM concentration and all the parameters were measured after 24, 48 respectively 72 hours after incubation.

Cumulative gas volume was similar between treatments at 24 hours incubation, representing an accelerating trend at 48 hours. IgY antibodies addition significantly decreased the gas volume at 48 and 72 hours ($P <0.05$). At 72 hours of incubation, it can be seen that p-coumaric acid decreased the total gas volume ($P <0.05$) when compared with control (43.42 ml/100 ml vs 36.27 ml/100 ml).

Addition of phenolic acids and IgY antibodies significantly ($P <0.05$) decreased methane production at 24 and 48 hours of incubation (Table 1). Caffeic acid had the higher inhibition rate (37.58%), followed by p-coumaric acid with an inhibition rate of 28.33% and IgY antibodies which had 26.69% inhibition rate. By 72 hours, the methane decrease was no longer evident ($P >0.05$). On the contrary, the IgY antibodies increased ($P <0.05$) the methane production when compared with control sample (12.74 ml/100 ml vs 11.85 ml/100 ml)

The results for volatile fatty acids production and pH of in vitro ruminal cultures are presented in Table 2. Caffeic and p-coumaric acids addition significantly decreased ($P <0.05$) propionate production at 48 hours of incubation. A decrease can also be observed for n- Butyrate in samples where simple phenols were added. Caffeic acid addition significantly decreased ($P <0.05$) iso-Butyrate and iso-Valerate production at 48 hours of incubation. An increase can be observed for both Caffeic and p-coumaric acids addition at 72 hours sampling ($P <0.05$).

IgY antibodies addition at ruminal cultures showed a significantly increase ($P <0.05$) for n- and iso-Butyrate at 24 hours (Table 2). Also, for the other VFA-s, IgY antibodies tend to increase the production, but the results were not significant ($P > 0.05$).

Addition of caffeic acid, p-coumaric acid and IgY antibodies had no effect ($P > 0.05$) on the pH results (Table 2). The results for volatile fatty acids production and pH of in vitro ruminal cultures are presented in Table 2. Caffeic and p-coumaric acids addition significantly decreased ($P <0.05$) propionate production at 48 hours of incubation. A decrease can also be observed for n- Butyrate

**Table 1.** Effects of simple phenols and of IgY avian-antibodies addition on cumulative gas volume and methane production of *in vitro* ruminal cultures

| Parameter | Time (h) | Treatments | | | | SD |
|---|---|---|---|---|---|---|
| | | Control | Caffeic acid | p-Coumaric acid | IgY | |
| Cumulative gas (ml/100 ml) | 24 | 35.87$^{a}$ | 34.12$^{a}$ | 34.12$^{a}$ | 36.49$^{a}$ | 1.76 |
| | 48 | 65.24$^{a}$ | 63.03$^{a}$ | 60.89$^{a}$ | 52.89$^{b}$ | 3.02 |
| | 72 | 43.42$^{a}$ | 39.05$^{ab}$ | 36.27$^{b}$ | 38.51$^{ab}$ | 1.97 |
| Methane (ml/100 ml) | 24 | 4.87$^{a}$ | 3.04$^{c}$ | 3.49$^{bc}$ | 3.57$^{b}$ | 0.17 |
| | 48 | 8.07$^{a}$ | 6.48$^{b}$ | 7.05$^{b}$ | 7.12$^{b}$ | 0.35 |
| | 72 | 11.85$^{ab}$ | 11.10$^{b}$ | 11.25$^{ab}$ | 12.74$^{a}$ | 0.58 |

a,b Values within a row with different superscripts differ significantly at $P<0.05$.

**Table 2.** Effects of simple phenols and of IgY avian-antibodies addition on volatile fatty acids production and pH of *in vitro* ruminal cultures

| Parameter | Time (h) | Treatments | | | | SD |
|---|---|---|---|---|---|---|
| | | Control | Caffeic acid | p-Coumaric acid | IgY | |
| Acetate (mg/l) | 24 | 1612.39[a] | 1587.82[a] | 1526.44[a] | 1705.80[a] | 79.93 |
| | 48 | 2175.44[a] | 2029.52[a] | 2056.65[a] | 2076.52[a] | 105.69 |
| | 72 | 2192.09[a] | 2235.29[a] | 2180.88[a] | 2247.73[a] | 113.74 |
| Propionate (mg/l) | 24 | 650.97[a] | 616.30[a] | 585.14[a] | 659.49[a] | 32.12 |
| | 48 | 763.04[a] | 644.55[b] | 668.31[b] | 693.08[ab] | 3.41 |
| | 72 | 739.84[a] | 702.87[a] | 695.04[a] | 730.81[a] | 35.21 |
| n-Butyrate (mg/l) | 24 | 507.76[ab] | 500.46[ab] | 450.96[b] | 526.98[a] | 24.22 |
| | 48 | 699.03[a] | 607.35[b] | 637.37[ab] | 671.55[ab] | 32.53 |
| | 72 | 680.25[a] | 666.71[a] | 667.66[a] | 721.26[a] | 33.95 |
| iso-Butyrate (mg/l) | 24 | 2.41[c] | 3.58[ab] | 3.96[a] | 3.44[b] | 0.16 |
| | 48 | 10.87[a] | 7.06[b] | 8.20[b] | 11.68[a] | 0.48 |
| | 72 | 18.97[b] | 19.46[b] | 24.32[a] | 15.83[c] | 0.95 |
| iso-Valerate (mg/l) | 24 | 32.27[a] | 22.90[b] | 34.62[a] | 33.76[a] | 1.55 |
| | 48 | 62.22[a] | 65.25[a] | 68.70[a] | 67.70[a] | 3.21 |
| | 72 | 103.49[a] | 105.85[a] | 111.41[a] | 106.06[a] | 5.40 |
| pH | 24 | 6.76[a] | 6.76[a] | 6.71[a] | 6.72[a] | 0.32 |
| | 48 | 6.71[a] | 6.61[a] | 6.64[a] | 6.59[a] | 0.33 |
| | 72 | 6.51[a] | 6.53[a] | 6.45[a] | 6.51[a] | 0.31 |

a,b Values within a row with different superscripts differ significantly at P<0.05.

in samples where simple phenols were added. Caffeic acid addition significantly decreased (P <0.05) iso-Butyrate and iso-Valerate production at 48 hours of incubation. An increase can be observed for both Caffeic and p-coumaric acids addition at 72 hours sampling (P <0.05).

IgY antibodies addition at ruminal cultures showed a significantly increase (P <0.05) for n- and iso-Butyrate at 24 hours (Table 2). Also, for the other VFA-s, IgY antibodies tend to increase the production, but the results were not significant (P > 0.05).

Addition of caffeic acid, p-coumaric acid and IgY antibodies had no effect (P > 0.05) on the pH results (Table 2).

Phenolic acids occur naturally as hydroxycinnamic acids and are present in almost all forage fed to ruminants (GULCIN [43], JAYANEGARA [44]) and other plant products (CLIFFORD [45]). They act as antimicrobial agents against fungi, bacteria and protozoa by intruding into the cell membrane to disintegrate its structures (BODAS & al. [46]). They have certain effects on the activity of ruminal microorganisms that depend first on the type of plant species that is consumed and second, on the chemical composition of the plant (BODAS & al. [46]).

In the present study both phenolic acids used had effects on ruminal parameters that were measured. Caffeic and p-coumaric acids were added in 12 mM concentration to ruminal liquid and both decreased the methane production in vitro at 24 and 48 hours of incubation. It has been observed that certain phenolic acids have a toxic effect on ruminal bacteria, on fungi and protozoa (LIM & al.

[47]). The process of inhibition of cell wall degrading microorganisms in rumen fluid by phenolic acids is not yet biochemically understood. The inhibition may be caused by damaging the cell membranes, and also by inactivation of cell enzymes (HARTLEY & AKIN [48]). As a result, the effect on ruminal methanogens could be expected and the methane decrease could be linked to their role in fibre degradation and in decreasing ruminal protozoa (JAYANEGARA [44]). In rumen, a large number of ruminal methanogens is attached to protozoa (WANG & al. [49], HESS & al. [50]) and this association contributes with up to 37% of total rumen methane emissions (KLIEVE & HEGARTY [51]). Therefore, a decrease in methane production can be associated with a reduction of protozoal counts.

The effects of simple phenols on rumen micro-organisms is concentration and source dependent. JAYANEGARA [44], used six sources of phenols, among them caffeic and p-Coumaric acids. In this study, the two simple phenols were added to ruminal cultures in two different concentration (5 and 10 mM/), and the results showed that both phenols decreased the methane and gas production in vitro when applied in 10 mM concentration. Methane decrease was relatively small, and the effects of phenols may depend on the concentration applied and, on the source (JAYANEGARA [44]). In a similar experiment, we used a series of four plant secondary metabolites, among them these two simple phenols tested in the current experiment (caffeic and p-coumaric acids) (GIUBURUNCA & al. [52]). The concentration tested was 6 mM and the results showed a decrease in total gas and methane production, but it was not significant ($p > 0.05$). It was showed that addition of p-coumaric acid 0.1% had the most toxic effect on the growth of *R. albus* and *R. flavefaciens* (VAREL & JUNG [53]). In another experiment, addition of p-coumaric at 1 mM retained almost 100% of cellulolytic activity of *B. succinogenes*, *R. flavefaciens*, *R. albus*, and when the concentration was increased (5 mM and 10 mM) the cellulolytic activity retained was 80% and 40%, respectively (CHESSON & al. [54]). These results showed that the effects of phenolic acids are dependent on the concentration used.

The decrease in methane and gas production was observed only at 24 and 48 hours of incubation in our experiment. After this period, this effect was not observed, and this might be explained by the adaptation of rumen microorganisms in the presence of phenolic acids. One mechanism of defense of certain microorganisms active in fiber degradation may be the hydrogenation of the more toxic phenolic acids to a less toxic form (JAYANEGARA [44], VAREL & JUNG [53], CHESSON & al. [54]). Also, phenolic acids can be lost from rumen fluid by non-specific absorption or by specific utilization by certain rumen microbes (AKIN [55], JAYANEGARA [44]).

In addition to simple phenols, we tested in our current experiment the avian IgY antibodies. These immuno-globulins are specific anti-methanogen antibodies and were tested for their efficacy on inhibiting methane production. The results showed that the addition of avian IgY antibodies to ruminal cultures decreased the methane production at 24 and 48 hours of incubation, but they also had an effect on VFAs concentration. The ideal inhibition strategy for methanogens would decrease total methane production without altering the other ruminal parameters, such as volatile fatty acid profile or fermentation. In a similar experiment, COOK & al. [40] suggest that specific anti-methanogen antibodies can be effective for inhibition of methanogenesis during ruminal fermentation process, but given the transient nature of fermentation, their effect is difficult to predict.

## Conclusions

In our experiment, the temporary effect on inhibition may be associated with the growth of non-culturable methanogens that were not sensitive to the antibodies present in the egg yolk. The mechanisms by which antibodies neutralize the methane production may be inhibiting growth of methanogens, agglutination of cells, inhibition of symbiotic interactions (COOK & al. [40]). Another factor that needs to be considered in order to achieve a continual methane reduction and to develop a passive immunization strategy, is the stability of egg antibodies (LI & al. [56], COOK & al. [40]).

## Acknowledgements

The authors are grateful to DBU (Deutsche BundesstiftungUmwelt) for the scholarship program at Helmholtz Centre for Environmental Research, Leipzig, Germany.

## References

1. H. STEINFELD, P. GERBER, T.D. WASSENAAR, V. CASTEL, C. DE HAAN, Livestock's Long Shadow: Environmental issues and options. Food Agriculture Organization, Rome, Italy, (2006).
2. V. SEJIAN, R. LAL, J. LAKRITZ, T. EZEJI, Measurement and prediction of enteric methane emission. International Journal of Biometeorology, 55(1), 1-16, (2001).
3. RAHOVEANU J., A.T., MOCUTA, D.N., RAHOVEANU, M.M.T. Impacts of zootechnical

activities on environment and human health – a case study. Romanian Biotechnological Letters, 23(3), (2018).
4. F. FORABOSCO, Z. CHITCHYAN, R. MANTOVANI, Methane, nitrous oxide emissions and mitigation strategies for livestock in developing countries: A review. South African Journal of Animal Science, 47, 268-280, (2017).
5. S. KUMAR, A.K. PUNIYA, M. PUNIYA, S.S. DAGAR, S.K. SIROHI, K. SINGH, G.W. GRIFFITH, Factors affecting rumen methanogens and methane mitigation strategies. World Journal of Microbiology and Biotechnology, 25, 1557-1566, (2009).
6. M.G. LOZANO, Y. PEÑA GARCIA, K.A. AVENDAÑO ARELLANO, C.E. LÓPEZ ORTIZ, N. BALAGURUSAMY, Livestock Methane Emission: Microbial ecology and mitigation strategies. Livestock Science SelimSekkin, IntechOpen, 51-69, (2017).
7. P.H. JANSSEN, Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Animal Feed Science and Technology, 160, 1-22, (2010).
8. A. PATRA, T. PARK, M. KIM, Z. YU, Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. Journal of Animal Science and Biotechnology, 8-13, (2017).
9. Y.Q. GUO, W.L. HU, J.X. LIU, Methanogens and manipulation of methane production in the rumen. Wei Sheng Wu XueBao, 45, 145-148, (2005).
10. Y. LIU, W.B. WHITMAN, Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Annals of the New York Academy of Sciences, 1125, 171-89, (2008).
11. D. MORGAVI, E. FORANO, C. MARTIN, C. NEWBOLD, Microbial ecosystem and methanogenesis in ruminants. Animal, 4, 1024-36, (2010).
12. R.E. HUNGATE, W. SMITH, T. BAUCHOP, IDA YU, J.C. RABINOWITZ, Formate as an intermediate in the bovine rumen fermentation. Journal of Bacteriology, 102, 389-397, (1970).
13. M. ROTHER, J.A. KRZYCKI, Selenocysteine, pyrrolysine, and the unique energy metabolism of methanogenic archaea. Archaea, 453-642, (2010).
14. J.H. THIELE, M. CHARTRAIN, J.G. ZEIKUS, Control of interspecies electron flow during anaerobic digestion: role of floc formation in syntrophic methanogenesis. Applied and Environmental Microbiology, 54, 10–19, (1988).
15. M. LANGE, P. WESTERMANN, B.K. AHRING, Archaea in protozoa and metazoan. Applied Microbiology and Biotechnology, 66, 465-474, (2005).
16. R. SHARP, C.J. ZIEMER, M.D. STERN, D.A. STAHL, Taxonspecific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiology Ecology, 26, 71-78, (1998).
17. B.J. FINLAY, G. ESTEBAN, K.J. CLARKE, A.G. WILLIAMS, T.M. EMBLEY, R.P. HIRT, Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiology Letters, 117, 157-161, (1994).
18. S.E. HOOK, A.G. WRIGHT, B.W. MCBRIDE, Methanogens: methane producers of the rumen and mitigation strategies. Archaea, 2010, 11 pages, (2010).
19. K.A. JOHNSON, D.E. JOHNSON, Methane emissions from cattle. Journal of Animal Science. 73, 2483-2492, (1995).
20. N.C. FRIGGENS, J.D. OLDHAM, R.J. DEWHURST, Proportions of volatile fatty acids in relation to the chemical composition of feeds based on grass silage. Journal of Dairy Science, 8, 1331-1334, (1998).
21. S.S. LEE, J.T. HSU, H.C. MANTOVANI, J.B. RUSSELL, The effect of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5, on ruminal methane production *in vitro*. FEMS Microbiology Letters, 217, 51-55, (2002).
22. W.G. BERGEN, D.B. BATES, Ionophores: their effect on production efficiency and mode of action. Journal of Animal Science, 58, 1465-1483, (1984).
23. J.B. RUSSELL, H.J. STROBEL, Effect of ionophores on ruminal fermentation. Applied and Environmental Microbiology, 55, 1-6, (1989).
24. H. GUAN, K.M. WITTENBERG, K.H. OMINSKI, D.O. KRAUSE, Efficacy of ionophores in cattle diets for mitigation of enteric methane. Journal of Animal Science, 84, 1896-1906, (2006).
25. T.A. WOOD, R.J. WALLACE, A. ROWE, Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. Animal Feed Science and Technology, 152, 62-71, (2009).
26. A.K. RENUKA, S.A. PUNIA, P. SRINIVASULU, T.S. GOUD, A.K. SINGH, L. SHARMA, S. SAINI, R. DEVI, A. KUMAR, S.V. SINGH, R.C. UPADHYAY, Controlling methane emissions from ruminants employing bacteriocin. Climate Resilient Livestock & Production System, December 2013, Chapter 13, 140-153, (2013).
27. C. MARTIN, D.P. MORGAVI, M. DOREAU, Methane mitigation in ruminants: from microbe to the farm scale. Animal, 4, 351-365, (2010).
28. H. CLARK, Methane emissions from ruminant livestock; are they important and can we reduce them?. Proceedings of the New Zealand Grassland Association, 71, 73-76, (2009).

29. H. CLARK, F. KELLIHER, C. PINARES-PATIÑO, Reducing $CH_4$ emissions from grazing ruminants in New Zealand: challenges and opportunities. Asian-Australasian Journal of Animal Sciences, 24, 295-302, (2011).
30. H.D. HESS, L.M. MONSALVE, C.E. LASCANO, J.E. CARULLA, T.E. DIAZ, M. KREUZER, Supplementation of a tropical grass diet with forage legumes and *Sapindus saponaria* fruits: effect on *in vitro* ruminal nitrogen turnover and methanogenesis. Australian Journal of Agricultural Research, 54, 703-713, (2013b).
31. G. GOEL, H.P.S. MAKKAR, K. BECKER, Effects of *Sesbaniasesban* and *Carduuspycnocephalus* leaves and Fenugreek (*Trigonella foenumgraecum L.*) seeds and their extracts on partitioning of nutrients from roughage- and concentrate-based feeds to methane. Animal Feed Science and Technology, 147, 72-89, (2008b).
32. R. BODAS, S. LÓPEZ, M. FERNÁNDEZ, R. GARCÍA-GONZÁLEZ, A.B. RODRÍGUEZ, R.J. WALLACE, J.S. GONZÁLEZ, *In vitro* screening of the potential of numerous plant species as anti-methanogenic feed additives for ruminants. Animal Feed Science and Technology, 145, 245-258, (2008).
33. H.D. HESS, M. KREUZER, T.E. DIAZ, C.E. LASCANO, J.E. CARULLA, C.R. SOLIVA, A. MACHMÜLLER, Saponin rich tropical fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. Anim. Animal Feed Science and Technology, 109,79–94, (2003a).
34. K.A. BEAUCHEMIN, S.M. MCGINN, Methane emissions from beef cattle: effects of fumaric acid, essential oil, and canola oil. Journal of Animal Science, 84, 1489- 1496, (2006).
35. A.D. WRIGHT, P. KENNEDY, C.J. O'NEILL, A.F. TOOVEY, S. POPOVSKI, S.M. REA, C.L. PIMM, L. KLEIN, Reducing methane emissions in sheep by immunization against rumen methanogens, Vaccine, 22, 3976-3985, (2004).
36. D.N. KAMRA, N. AGARWAL, L.C. CHAUDHARY, Inhibition of ruminal methanogens by tropical plants containing secondary compounds. International Congress Series, 1293, 156-163, (2006).
37. T. HARTMANN, From waste products to eco-chemicals: Fifty years research of plant secondary metabolism. Phytochemistry, 68, 2831-2846, (2007).
38. R. BODAS, M. FERNÁNDEZ, R. GARCÍA-GONZÁLEZ, S. LÓPEZ, R.J. WALLACE, Phytogenic additives to decrease *in vitro* ruminal methanogenesis, T.G. Papachristou, Z.M. Parissi, H. Ben Salem, P. Morand-Fehr, eds,. Nutritional and foraging ecology of sheep and goats. Zaragoza: CIHEAM/FAO/NAGREF, 2009. Options Méditerranéennes: Série A. Séminaires Méditerranéens, 85, 279-283, (2009).
39. A. JAYANEGARA, Reducing Methane Emissions from Livestock: Nutritional Approaches. Dipresentasi-kanpada Indonesian Students Scientific Meeting (ISSM), Institute for Science and Technology Studies (ISTECS), European Chapter, May 13-15, Delft, the Netherlands, (2008).
40. S.R. COOK, K. MAITI, A.V. CHAVES, C. BEN-CHAAR, K.A. BEAUCHEMIN, T. MCALLISTER, Avian (IgY) anti-methanogen antibodies for reducing ruminal methane production: *in vitro* assessment of their effects. Australian Journal of Experimental Agriculture, 48, 260-264, (2008).
41. A. CRISTE, Research concerning influence of extraction method on quantity of IgY isolated from hen eggs. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, 61, 383-389, (2005).
42. P.M. FEDORAK, S.E. HRUDEY, A simple apparatus for measuring gas production by methanogenic cultures in serum bottles. Environmental Technology Letters,4, 425-432, (1983).
43. I. GULCIN, Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology, 217, 213-220, (2006).
44. A. JAYANEGARA, Ruminal methane production on simple phenolic acids addition in *in vitro* gas production method. Media Peternakan, 32, 53-62, (2009).
45. M.N. CLIFFORD, Chlorogenic acids and other cinnamates: nature, occurrence and dietary burden. Journal of the Science of Food and Agriculture, 79, 362-372, (1999).
46. R. BODAS, N. PRIETO, R. GARCÍA-GONZÁLEZ, S. ANDRES, F.J. GIRALDEZ, S. LÓPEZ, Manipulation of rumen fermentation and methane production with plant secondary metabolites. Animal Feed Science and Technology, 176, 78-93, (2012).
47. T.Y. LIM, Y.Y. LIM, C.M. YULE, Evaluation of antioxidant, antibacterial and anti-tyrosinase activities of four Macaranga species. Food Chemistry, 114, 594-599, (2009).
48. R.D. HARTLEY, D.E. AKIN, Effect of forage cell wall phenolic acids and derivatives on rumen microflora. Journal of the Science of Food and Agriculture, 49, 405-411, (1989).
49. C.J. WANG, S.P. WANG, H. ZHOU, Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. Animal Feed Science and Technology, 148, 157-166, (2009).

50. H.D. HESS, M. KREUZER, T.E. DIAZ, C.E. LASCANO, J.E. CARULLA, C.R. SOLIVA, A. MACHMÜLLER, Saponin rich tropical fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. Anim. Animal Feed Science and Technology, 109, 79-94, (2003).
51. A.V. KLIEVE, R.S. HEGARTY, Opportunities for biological control of ruminal methanogenesis. Australian Journal of Agricultural Research, 50, 1315-1319, (1999)
52. M. GIUBURUNCĂ, A. CRISTE, D. COCAN, R. CONSTANTINESCU, C. RĂDUCU, V. MIREȘAN, Effects of plant secondary metabolites on methane production and fermentation parameters in *in vitro* ruminal cultures. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, 47, 78-82, (2014).
53. V.H. VAREL, H.J.G. JUNG, Influence of forage phenolics on ruminalfibrolytic bacteria and *in vitro* fiber degradation. Applied and Environmental Microbiology, 52, 275-280, (1986).
54. A. CHESSON, C.S. STEWART, R.J. WALLACE, Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria. Applied and Environmental Microbiology, 44, 597-603, (1982).
55. D.E. AKIN, Attack on lignified grass cell walls by a facultatively anaerobic bacterium. Applied and Environmental Microbiology, 40, 809-820, (1980).
56. X. LI, T.A. MCALLISTER, K. STANFORD, J.Y. XU, Y. N. LU, Y.H. ZHEN, Y.X. SUN, Y.P. XU, Chitosan-alginate microcapsules for oral delivery of egg yolk immunoglobulin (IgY). Journal of Agricultural and Food Chemistry, 55, 2911-2917, (2007).