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Robert WALLACE, *et al*:
Fumaric Acid vs Methane

Fumaric acid feed supplement reduces animal methane emissions by 70% !
(Wild sources: angelica, common fumitory, shepherd's purse & bird's-foot trefoil)

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https://phys.org/news/2008-03-scientists-cow-flatulence.html
March 21, 2008

Scientists look to cut cow flatulence

Scientists in Scotland said they've found a way to cut greenhouse gas emissions by curbing cow and sheep flatulence.

The Rowett Research Institute in Aberdeen said 60 percent of global methane formation is due to agricultural activities, with just under half of that produced by ruminants such as cows and sheep, the Scottish government said Wednesday in a release.

Researchers said the average cow contributes as much to global warming as a family car that travels 12,000 miles, The Daily Telegraph reported.

The scientists found that adding fumaric acid to animal feed can inhibit the production of methane that occurs naturally as part of the animal's digestive process. Tests of the feed additive on lambs showed methane production can be cut by up to 70 percent. The feed also resulted in lambs gaining weight faster.

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https://www.theguardian.com/commentisfree/2018/aug/25/veganism-intensively-farmed-meat-dairy-soya-maize

If you want to save the world, veganism isn't the answer
Isabella Tree

Intensively farmed meat and dairy are a blight, but so are fields of soya and maize. There is another way...

Much has been made of the methane emissions of livestock, but these are lower in biodiverse pasture systems that include wild plants such as angelica, common fumitory, shepherd's purse and bird's-foot trefoil because they contain fumaric acid – a compound that, when added to the diet of lambs at the Rowett Institute in Aberdeen, reduced emissions of methane by 70%.

In the vegan equation, by contrast, the carbon cost of ploughing is rarely considered. Since the industrial revolution, according to a 2017 report in the science journal Nature, up to 70% of the carbon in our cultivated soils has been lost to the atmosphere.

So there's a huge responsibility here: unless you're sourcing your vegan products specifically from organic, "no-dig" systems, you are actively participating in the destruction of soil biota, promoting a system that deprives other species, including small mammals, birds and reptiles, of the conditions for life, and significantly contributing to climate change...

Feed Additive
WO2006040537

The present invention relates to the use of encapsulated organic acid(s), especially fumaric acid to decrease methane production in ruminants. There is also provided a ruminant feed composition which comprises encapsulated fatty acid(s), especially fumaric acid for use in decreasing methane production by ruminants. Such uses and compositions may also or alternatively lead to increased body mass and/or milk production by the ruminants.

IMPROVED RUMINANT FEEDING

Field of the invention

The present invention relates to the reduction of methane production in ruminants and/or improved meat and/or milk production. In particular the present invention relates to the use of encapsulated organic acid(s), especially fumaric acid to decrease methane production in ruminants. There is also provided a ruminant feed composition which comprises encapsulated fatty acid(s), especially fumaric acid for use in decreasing methane production by ruminants. Such uses and compositions may also or alternatively lead to increased body mass and/or milk production by the ruminants.

Introduction

Methane is an important greenhouse gas, contributing approximately 18% to the overall radiative forcing [1] and to global warming. This has been recognised globally and the Kyoto Protocol has global reductions for methane set at 5.2% below the 1990 level for the period 2008-2012[I]. Within the UK, the government has set even stricter reductions and is committed to reducing methane emissions to 12.5% below 1990 levels by the same period[2].

In Scotland, the largest source of methane is agriculture; contributing approximately 57%, which accounts for 17% of all the UK's agricultural emissions[3]. Pretty et al [4] estimated the financial damage to the UK over the period 1990-1996 from methane emissions to be [pound]280M per annum. Therefore, the cost to Scotland could be estimated at [pound]47.6M.

With the European Union discussing methods for taxing emissions of greenhouse gases, a technology that decreased methane in the agricultural sector would be in great demand.

The reason behind decreasing methane so far has been environmental but there are other

benefits. For the feed industry, methane production (methanogenesis) in the rumen represents a loss of energy in the growing animals. It is estimated that between 5 and 15% of dietary energy is lost as methane by eructation in ruminants[5]. Therefore, if this loss were prevented, the animals would require less feed.

Several compounds have been studied as potential feed additives for ruminants as a method of reducing methane production in the rumen[6], such as medium chain fatty acids[7, 8], oxaloacetate and butyrate enhancers[9], sodium fumarate[10, 11], oils[12, 13], organic acids [9, 14, 15] and fumaric acid[16]. Initial trials have shown that fumaric acid would act as an alternative hydrogen acceptor and prevent H2 reaching the methanogenic archaea, refer to Figure 1. Figure 1 shows how a methanogen could divert reducing equivalents, including hydrogen (illustrated by [2H] in Figure 1) produced by carbohydrate oxidation and generate methane and water. Alternatively, if fumaric acid (fumarate) or an alternative organic acid were added, then the reduction of fumarate to succinate could be increased, taking the reducing equivalents away from the methanogens and generating more carbon flow to propionate. This would lead to a decrease in methane produced by the methanogenic archaea. [17] However, there was evidence of a decrease in rumen pH associated with addition of free acids.

It is an object of the present invention to obviate and/or mitigate at least one of the aforementioned disadvantages.

In a first aspect there is provided a feed composition for ruminants comprising at least one encapsulated organic acid and/or salt thereof.

By feed composition is meant a composition, which is eaten by a ruminant and digested in the gastrointestinal tract. It is to be understood that said at least one encapsulated organic acid is generally provided in addition to or in combination with other conventional feed components. Conventional feed components may include a selection of the following: cereals such as corn, milo, wheat, barley, rye, oat, wheat flour, unpolished rice, millet, soybean, soybean flour, cassava, etc., oil meals such as soybean meal, dehulled soybean meal, rapeseed oil meal, peanut oil meal, linseed oil meal, sesame oil meal, coconut oil meal, sunflower oil meal, safflower oil meal, palm kerned oil meal, kapok oil mean, etc.; feeds of animal origin such as fish meal, fish solubles, meat scrap, meat-and-bone meal, blood meal, feather meal, silkworm cocoon oil meal, skimmed milk, whey, animal oils (e.g. beef oil, lard oil, bone oil, etc.), brewers' yeast, torula yeast, etc.; mineral feeds such as sodium chloride, calcium sources (e.g. calcium carbonate, limestone powder, oyster shell, etc.) and phosphorous sources (e.g. dicalcium phosphate, tricalcium phosphate, etc.); vitamins, amino acids and minerals. However, this is understood not to include animals grazing on grass, or eating roughage alone, when seed feed composition is provided separately.

The feed, if necessary, may contain a variety of additives such as an antibiotic, preservative, enzyme, anti-fungal agent, antioxidant, colorant, sweetener, perfume, binder and so on. In the feed, cereals are contained generally in a proportion of about 30 to 80% by weight and preferably about 40 to 80% by weight.

The feed is frequently given with a roughage. The roughage is primarily composed of cellulosic materials such as plant stems and leaves, e.g. alfalfa meal, timothy hay, introduced grass, native grass, green roughage, straw, tree leaves, etc., brans such as rice bran, barley bran, wheat bran, etc. and crude fibers (e.g. factory byproducts such as gluten food, gluten

meal, starch meal, molasses, soy sauce byprodcuts, brewery's byproducts, beat pulp, bagasse, soybean curd cake, malt sprouts, mandarin orange peels, mandarin orange juice cake, etc.

The feed compositions according to the present invention comprise an encapsulated organic acid, or salt thereof. The organic acid may be pyruvic acid, acrylic acid, aspartic acid, malic acid, citric acid or tartric acid but is preferably fumaric acid. Suitable salts include, for example, salts with alkali or alkaline earth metals, e.g. potassium, sodium, calcium, barium, magnesium, and ammonium. The present invention also includes the use of mixtures of said organic acid and/or any of said salts and may therefore include the use of one or more acids in combination with one or more salts.

Typically the amount of said at least one organic acid, especially fumaric acid, and/or salt(s) thereof, will be from about 1% - 20% w/total weight of feed composition, such as 10% - 20%, preferably 7.5% - 15%. It is envisaged that a total amount of approximately 10 g to 250 g per day may be ingested by a sheep/goat or up to about 100 g to 2.5 kg for cattle.

The feed composition of the present invention can be manufactured by conventional means. For example, said at least one encapsulated organic acid may be blended with the other feed components and moulded, if appropriate, into granules, pellets, cakes and the like. Alternatively the feed composition comprising encapsulated organic acid and/or salt thereof may be formulated into granules, pellets, cakes and the like and optionally mixed with other feed components.

Said at least one organic acid or salt thereof is/are encapsulated such that upon mechanical, chemical or enzymic disruption of the feed composition, as occurs due to, for example, chewing and/or mixing in the rumen, said organic acid or salt thereof generally remains coated and/or otherwise associated with a suitable encapsulating agent. It is intended that said organic acid or salt thereof should be substantially encapsulated when entering the rumen. It is to be understood therefore that the term "encapsulated" does not refer merely to combining with conventional feed components and, for example, forming into pellets or cakes. Thus, said at least one organic acid and/or salt thereof, is first encapsulated within a suitable encapsulating agent and thereafter mixed with said other feed additives. The term "encapsulated" is understood to relate to compositions which generally comprise an inner organic acid and/or salt thereof core and an outer encapsulating agent layer, as well as compositions in which the organic acid and/or salt thereof may be distributed within an encapsulating matrix, emulsion, body, substrate or the like.

Preferably said at least one organic acid and/or salt thereof may be encapsulated within a material which is poorly, or slowly soluble/broken down/digested in the rumen. In this manner, said at least one organic acid and/or salt may be released into the rumen over an extended period of time, such as 2-24 hours, e.g. 4 to 8 hours. Thus, in a further aspect, there is provided a slow release ruminant feed component, said component comprising at least one organic acid and/or salt thereof.

Slow release is intended to cover compositions which release said organic acid and/or salt thereof, over a number of hours, 2-24 hours (e.g. 4 to 8 hours) and/or release said organic acid and/or salt thereof in such a manner that the pH of the rumen does not fall below pH 6.

In this manner, it is possible to ensure that low concentrations of said organic acid and/or salt thereof are present in the rumen, over a period of time. Typically said organic acid and/or salt

thereof may be encapsulated in a lipid coating, such as a mono-, di- and/or tri-glyceride, oils, such as hydrogenated or partially hydrogenated vegetable oil, coconut oil, palm oil, waxes, organic esters or combinations thereof. Alternatively, said organic acid and/or salt thereof may be coated in a natural or synthetic polymer that is capable of allowing slow release of said organic acid and/or salt thereof, into the rumen. Examples of suitable polymers include hydroxyalkyl carboxylate polyester, cellulose or amylose based polyers, polyethylene glycol, polyvinyl pyrrolidone and polyhydroxyalkamoate.

Particularly suitable encapsulating agents and formulations are described in US6,312,741, to which the skilled reader is directed. Preferred compositions of the present invention comprise an encapsulating agent in an amount of about 10% - 50% w/total weight of the composition and 90% - 50% w/total weight of the composition organic acid and/or salt thereof. Particularly preferred compositions are marketed under the trademark Bakesure(R), such as Bakeshure 451 and Bakeshure 470, which have the following compositions:

Bakeshure<(R)> 451
Fumaric acid 83% to 87%
Coated with partially hydrogenated vegetable oil.
Coating content 13% to 17%.
Bakeshure (R) 470
Fumaric acid 61% to 65%.
Coated with partially hydrogenated vegetable oil.
Coating content 35% to 39%.

Advantageously, the present inventors have observed that encapsulating said at least one organic acid or salt thereof does not lead to such an undesirable pH drop in the rumen, as observed when using unencapsulated organic acid, such as fumaric acid and leads to more consistent inhibition of methane formation. Additionally, more feed comprising the encapsulated organic acid or salt thereof (e.g. fumaric acid) is ingested by a ruminant. Without wishing to be bound by theory, increased feeding may be due to the lesser reduction in pH in the rumen and/or the feed being more palatable to the ruminant.

It is envisaged that the feed compositions according to the present invention can result in decreased methane production and/or increased/improved productivity, such as increased and/or better quality milk and/or meat production. Specifically, the altered fatty acid composition of milk and meat may promote improved health in man.

Thus, in a further aspect, there is provided use of an organic acid and/or salt thereof, especially fumaric acid and/or salt thereof in the manufacture of an encapsulated and/or slow release formulation for reducing methane production and/or increasing/improving the quality of milk and/or meat produced by said ruminant.

In a further aspect there is provided a method of reducing methane production and/or increasing/improving the quality of milk and/or meat produced by a ruminant comprising the step of feeding to a ruminant an encapsulated or slow release organic acid and/or salt thereof.

The present invention will now be further described by way of example and with reference to the figures, which show:

Figure 1 shows the mode of action of an organic acid in decreasing methane formation.

Figure 1

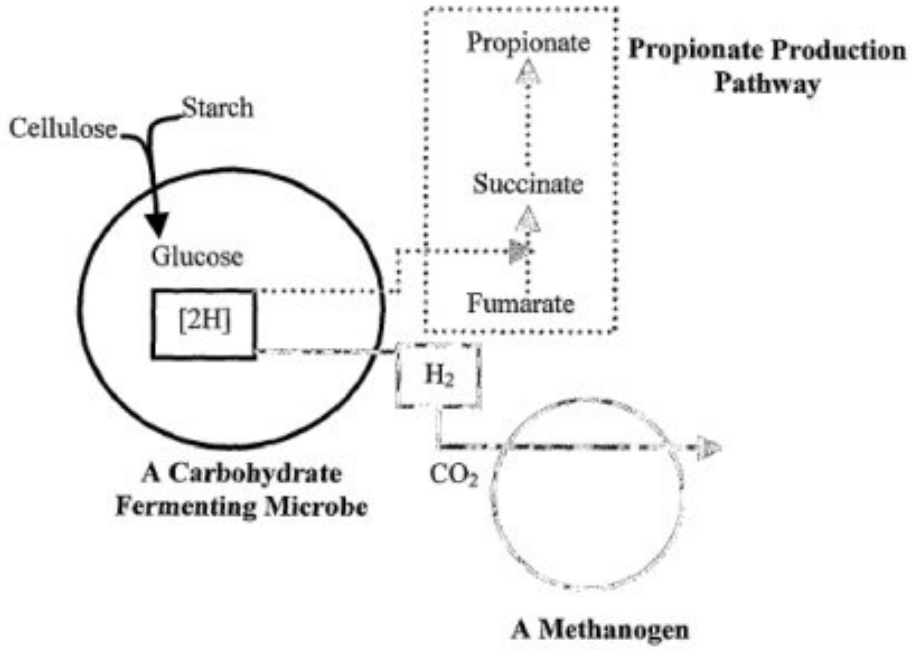

Figure 2 shows the effect of fumaric acid addition on the increase in total volatile fatty acid concentration ([mu]moles/50 mL) in vitro.

Figure 3 shows the effect of fumaric acid addition on the increase in propionate concentration ([mu]moles/50 mL) in vitro.

Figure 4 shows the effect of fumaric acid addition on propionate production rate ([mu]moles/hr) in vitro.

Figure 5 shows the effect of encapsulated fumaric acid and fumaric acid on the pH of unbuffered rumen fluid in vitro.

Figure 6 shows the effect of fumaric acid additives added to the diet on rumen pH in vivo.

Figure 7 shows the effect of fumaric acid additives added via a cannula on rumen pH in vivo.

Figure 8 shows an estimation of feed remaining after fumaric acid has been added to the diet .

Figure 9 shows the effect of additives added to the diet on propionic acid concentration in vivo.

Figure 10 shows the effect of additives added via the cannula on propionic acid in vivo.

Figure 11 shows the effect of different forms of encapsulated fumaric acid that can be effective.

Materials and Methods

The Effect of Free Fumaric Acid and the Salt (Sodium Fumarate) on Methane and Volatile Fatty Acid Production

A 0.4 M solution of sodium fumarate was used, prepared in distilled water, and 1 mL (0.4 mmoles sodium fumarate) added to 5 bottles containing 400 mg GP diet (grass hay, rolled barley, cane molasses, fish meal and minerals and vitamins (Lamscov Intensive Lamb 317, Norvite, Insch, Aberdeenshire); 500, 299.5, 100, 91 and 9.5 g/kg dry matter respectively) to give a final concentration of 8 mM sodium fumarate. A 0.2 M solution of fumaric acid was used (due to low solubility), prepared in distilled water, and 1 mL (0.2 mmoles fumaric acid) added to 5 bottles containing 400 mg GP diet to give a final concentration of 4 mM fumaric acid. Another 5 bottles received 1 mL of distilled water (controls). After 24 hours of incubation, analyses of methane, hydrogen, carbon dioxide and nitrogen were carried out.

The Effect of Rate of Addition of Sodium Fumarate on Methane and Volatile Fatty Acid Analysis

A 0.4 M solution of sodium fumarate was used, prepared in distilled water, and 1 niL (0.4 mmoles sodium fumarate) added to 5 bottles containing 400 mg General Purpose (GP) diet to give a final concentration of 8 mM sodium fumarate. Another 10 bottles received 1 mL of distilled water (controls and timed addition samples). Then at 6 hourly intervals 0.5 mL distilled water was added to the control samples and the bottles containing 0.4 M sodium fumarate. A 0.133 M solution of sodium fumarate was used, prepared in distilled water, and 0.5 mL (0.133 mmoles sodium fumarate) added to 5 bottles containing 400 mg GP diet + 1 mL distilled water at 6 hourly intervals to give a total of 0.399 mmoles sodium fumarate.

After 6 hours of incubation, analyses of methane, hydrogen, carbon dioxide, nitrogen and volatile fatty acids were carried out.

The Effect of Encapsulated Fumaric Acid on Methane and Volatile Fatty Acid Production

Encapsulated fumaric acid (Bakeshure(TM) 451, Balchem), 83% to 87%, was used for the following experiment. 0 mg, 11.8 mg (equivalent to 10.030 mmoles fumaric acid), 17.6 mg (equivalent to 0.123 mmoles fumaric acid), 35.5 mg (equivalent to 0.260 mmoles fumaric acid) and 54.6 mg (equivalent to 0.400 mmoles fumaric acid) encapsulated fumaric acid were added to Wheaton bottles containing 400 mg general purpose feed in triplicate. For comparison, three bottles containing 400 mg general purpose feed and 46.4 mg fumaric acid were also set up. Another three bottles were set up containing 400 mg general purpose feed only (controls).

After 24 hours of incubation, analyses of methane, hydrogen, carbon dioxide, nitrogen and volatile fatty acids were carried out. The Effect of Encapsulated Fumaric Acid and Fumaric Acid on the Generation Rate of Volatile Fatty Acids

Encapsulated fumaric acid (Bakeshure(TM) 451), 83% to 87%, was used for the following experiment. 54.6 mg (equivalent to 0.400 mmoles fumaric acid) encapsulated fumaric acid was added to five Wheaton bottles containing 400 mg general purpose feed. 46.4 mg (equivalent to 0.400 mmoles) fumaric acid was added to five Wheaton bottles containing 400

mg general purpose feed. Another five bottles were prepared containing 400 mg general purpose feed only (controls). After 1, 2, 3, 4, 5, 22 and 24 hours of incubation, volatile fatty acids analysis was carried out.

The Effect of Encapsulated Fumaric Acid and Fumaric Acid on the pH of Unbuffered Rumen Fluid

The following experiment used distilled water in place of the buffer described in Section 2.1. Encapsulated fumaric acid (Bakeshure(TM) 451), 83% to 87%, was used for the following experiment. 54.6 mg (equivalent to 0.400 mmoles fumaric acid) encapsulated fumaric acid was added to five Wheaton bottles containing 400 mg general purpose feed. 46.4 mg (equivalent to 0.400 mmoles) fumaric acid was added to five Wheaton bottles containing 400 mg general purpose feed. Another five bottles were prepared containing 400 mg general purpose feed only (controls). After 1, 2, 3, 4, 5, 22 and 24 hours of incubation, pH measurements were taken using a Russell 660 pH meter.

The Effect of Fumaric Acid, Bakeshure<(R)> 451 and Bakeshure<(R)> 470 on the pH and Volatile Fatty Acid Concentration of Sheep Rumen Fluid In Vivo.

Four Dorset/Suffolk crossed sheep (Identity Numbers 2372, 948, 979 and 2988) housed and fed individually were used. The experimental design involved a (4 x 4) balanced Latin square as described below:-

Sheep Number
Day 1 2 3 4
1 A B C D
2 B D A C
3 C A D B
4 D C B A A = 75 g Fumaric Acid - MW 116.1

B = 75 g Encapsulated Fumaric Acid - Bakeshure<(R)> 470, 61% to 65% fumaric acid. C = 75 g Encapsulated Fumaric Acid - Bakeshure<(R)> 451, 83% to 87% fumaric acid. D = Control

The random number command in Excel was used to allocate the sheep identification tags to numbers 1, 2, 3 and 4. Sheep 1 = 979 Sheep 2 = 2988 Sheep 3 = 2372 Sheep 4 = 948

Week 1 - Addition of Additives Via the Diet

Animals were fed 400 g of ewe lamb feed (EL) (EWE LAMB DIET

Hay 300kg Barley 422.5kg Hypro Soya 167.5 Molasses 100kg Salt 3.5kg

Dical/ Phosphate 2.5kg Limestone 2.5kg Mins/vits 1.5kg In 1000kg mix)

plus 75 g of the relevant supplement or 475 g EL only if in the control group on the morning of the experiment. Supplements were mixed thoroughly with the EL feed in the feed tray before being given to the animals and water was freely available.

Week 2 - Addition of Additives Via the Cannula

75 g of each additive was added to a sample bottle and approximately 200 ml rumen fluid added. A rod was used to mix the additive with the rumen fluid. The rumen fluid was then poured back into the sheep rumen using the cannula.

Animals were fed 400 g of ewe lamb feed (EL) or, 475 g EL feed only if in the control group on the morning of the experiment.

Each period of the Latin square consisted of feeding each supplement then extraction of a 20 mL rumen fluid sample via a cannula at the following times: -pre-feeding, 1/2 hour after feeding and then again at 1 hr, 2 hrs, 4 hrs and 7 hrs post feeding. At each sampling time, an estimation of the feed remaining was recorded. The sheep were not fed again until after the last samples were taken.

A 3 day rest period between Latin Squares was incorporated into the experiment, where the animals received 1000 g ewe lamb feed over two servings, a.m. and p.m. for one day and 1200 g ewe lamb feed over two servings, a.m. and p.m. for the remaining two days.

Upon transfer of samples to the laboratory, the rumen fluid was strained through a double layer of gauze into a sterile universal bottle. 4 mL of each sample was transferred to a reaction tube containing 1 mL 20% orthophoshoric acid containing 20 mM 2-ethyl butyric acid and stored for VFA analysis. The pH of the remaining sample was then recorded.

Encapsulation of Fumaric Acid

1.5 g of the selected oil was melted in an 800W Hinari (Lifestyle) microwave oven on FULL power for 2 minutes and stirred. The mixture was returned to the microwave for a further 30 seconds until the liquid was clear. 8.5 g Sigma-Aldrich fumaric acid was placed in an IKA AlO electric mill. The melted oil was drizzled over the fumaric acid powder, and the fumaric acid (85%) and oil (15%) mixture were blended for approximately 30 seconds. A stainless steel spatula was used to remove any powder stuck to the sides or lid and the process repeated 2 or 3 times until the fumaric acid had been thoroughly mixed with the oil.

Analytical Methods

Gas Analysis

Total gas was measured using a 100 mL glass syringe connected to a 0.5 x 16 mm needle, which was injected through the stoppers into the headspace. A gas sample (1 mL) was removed from each bottle and analysed for methane, hydrogen, nitrogen and carbon dioxide by gas chromatography using a PYE Unicam GCV. The column used was a 4 mm x 3 m glass column packed with Porapak Q mesh 60-80 (Waters Associates Inc., Milford, MA, USA). Detector temperature: 150[deg.]C, injector temperature was: 85[deg.]C and the carrier gas (argon) flow rate was 30 mL/min; a katherometer detector was used. Peaks were identified by comparison with gas standards of known composition.

Volatile Fatty Acid Analysis

Sample fluid (4 mL) was added to 1 mL of an acid solution containing 20% orthophosphoric acid containing 20 mM 2-ethyl butyric acid as the standard. Samples were centrifuged at 14 000 rpm (20 000 - 24 000 x g) for 15 minutes at 4<0>C using a Sorvall RC-SB refrigerated

superspeed centrifuge. Volatile fatty acids were determined using a Hewlett Packard 5890 Series II Gas Chromatograph in accordance with the method described by Stewart and Duncan[18].

Statistical Analyses

Differences between the groups for each experiment were calculated using a single variation ANOVA in an Excel worksheet. P values were described as either: - Not Significant (NS), P > 0.05 Significant (*), P between 0.01 and 0.05 Very Significant (**), P between 0.001 and 0.01 Extremely Significant (***), P < 0.001

Results

Example 1: The Effect of Free Fumaric Acid and the Salt (Sodium Fumarate) on Methane and Volatile Fatty Acid Production

See above for methods. Results are shown in Table 1. Methane production decreased in the presence of sodium fumarate and fumaric acid. Although it appears sodium fumarate has reduced methane production by twofold compared to fumaric acid, the concentration of sodium fumarate was twice that of the fumaric acid. Therefore, the difference between the effect of sodium fumarate and fumaric acid upon methane production is small. If it were assumed that the concentration of fumaric acid is directly linked to methane production then methane would be decreased by 15.3% for a 0.4 M solution. This can be compared with a 14.2% reduction in methane produced by a 0.4 M solution of sodium fumarate. Table 1. The Effect of Free Fumaric Acid and the Salt (Sodium Fumarate) on Methane and Volatile Fatty Acid Production

Methane* C2<NS> C3<NS> C4<NS> Total VFA<NS>

([mu]mol/d) ([mu]mol/d) ([mu]mol/d) ([mu]mol/d) ([mu]mol/d)

Control 860.8 1670.7 648.8 383.6 2778.3

Sodium 738.6 1427.3 798.0 298.3 2581.7

Fumarate

Fumaric Acid 794.8 1464.4 720.7 323.8 2572.3 * Results varied significantly between groups. <NS> There was no significant difference between the groups.

No effect was observed in volatile fatty acid production.

Example 2: The Effect of Rate of Addition of Sodium Fumarate on Methane and Volatile Fatty Acid Analysis

See above for methods. Results are shown in Table 2. Methane production was less in the complete addition and timed addition samples than the control but statistical analyses showed no significant differences between the three groups. This could be due to the small volumes of gas produced over 6 hours. Propionate production increased in the presence of sodium fumarate but was higher when added in batches rather than in bulk at the start of the

incubation.

Table 2. The Effect of Rate of Addition of Sodium Fumarate on Methane and Volatile Fatty Acid Analysis
Methane<NS> C2<NS> C3** C4<NS> Total VFA<NS>
([mu]mol/h) ([mu]mol/h) ([mu]mol/h) ([mu]mol/h) ([mu]mol/h) Control 53.7 136.5 65.3 20.8 226.3
Complete 50.0 125.8 81.2 14.1 223.9 Addition
Timed 49.7 130.4 100.8 15.2 249.3
Addition

** Results varied very significantly between groups. <NS> There was no significant difference between the groups. Example 3: The Effect of Encapsulated Fumaric Acid on Methane and Volatile Fatty Acid Production

See above for methods. Results are shown in Table 3. Methane production decreased in the presence of encapsulated fumaric acid (EFA) and fumaric acid (FA). It must be noted that a high degree of variance was observed in the methane results from groups 17.6 mg EFA and 35.3 mg EFA. Propionate production (C3) increased with addition of encapsulated fumaric acid, as did the total volatile fatty acid production.

Table 3. The Effect of Encapsulated Fumaric Acid on Methane and Volatile Fatty Acid Production
Methane** C2** C3*** C4** <T>ota<l> VFA- *
([mu]mol/d) ([mu]mol/d) ([mu]mol/d) ([mu]mol/d) ([mu]mol/d)
Control 695.6 1251.9 466.1 288.1 2069.0
11.8 mg EFA 748.8 1131.1 452.1 261.6 1900.2
17.6 mg EFA 763.7 1216.8 494.1 272.0 2040.4
35.3 mg EFA 630.2 1323.3 556.5 308.4 2252.7
54.6 mg EFA 645.4 1519.8 662.2 351.5 2604.0
46.4 mg FA 640.4 1524.4 752.1 340.3 2683.9
*** Results varied extremely significantly between groups. ** Results varied very significantly between groups.

The percentage of methane depletion observed at 54.6 mg EFA and 46.4 mg FA was slightly higher in fumaric acid samples (7.9%) than in encapsulated fumaric acid samples (7.2%). This may have been due to the effects of the vegetable oil layer surrounding the encapsulated fumaric acid. Unexpectedly, at low concentrations of EFA (11.8 mg and 17.6 mg), methane production increased and volatile fatty acid generation decreased when compared with the control. Again, this could be due to unknown effects introduced by the vegetable oil, which are overcome at higher concentrations. A greater increase in propionate production was observed in samples containing fumaric acid, 46.4 mg (61.1%) than EFA, 54.6 mg (42%). It is possible that not all the encapsulated fumaric acid had dissolved and was still being released into the in vitro system at the end of the 24 hour period.

Example 4: The Effect of Encapsulated Fumaric Acid and Fumaric Acid on the Generation Rate of Volatile Fatty Acids

Results are shown in Figures 2, 3 & 4, Tables 4 & 5 and Appendix I.

Total volatile fatty acid concentration increased slightly with addition of encapsulated fumaric acid and fumaric acid, see Figure 2. No significant differences between the experimental groups were observed for acetic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid and caprioic acid. However, there were significant differences between the experimental groups for propionic acid concentration and propionic acid production rate, see Figures 3 and 4.

Initially, fumaric acid appeared to inhibit propionic acid production, but after 4 hours this effect disappeared and the propionic acid concentration and production rate remained just slightly above that of the EFA groups.

Encapsulated fumaric acid also increased propionic acid concentration and production rate, but the increase was more stable than that seen in fumaric acid groups.

Table 4. The Effect of Encapsulated Fumaric Acid on Volatile Fatty Acid Production

Total

C2<NS> C3*** C4<NS> VFA<NS>

([mu]mol/d) ([mu]mol/d) ([mu]mol/d) ([mu]mol/d) Control 2101.1 670.3 478.6 3356.5 EFA 2050.9 904.6 458.0 3515.5 FA 2106.6 946.5 504.3 3664.1

*** Results varied extremely significantly between groups. <NS> There was no significant difference between the groups. EFA is encapsulated fumaric acid and FA is fumaric acid.

After 24 hours, there was a significant difference in the [mu]moles propionic acid produced, see Table 4. Although the encapsulated fumaric acid samples did not generate the same number of [mu]moles as the fumaric acid samples, encapsulation has certainly not inhibited propionic acid production.

Table 5. Conversion (%) of Propionic Acid by Feed Additives and Potential [mu]moles Captured

Conversion to Potential [mu]moles Propionic Acid Methane Captured by

% Propionic Acid

Encapsulated Fumaric Acid 58.6 330

Fumaric Acid 69.1 340

After 24 hours, samples containing fumaric acid had converted 69.1% to propionic acid whereas those containing encapsulated fumaric acid showed a 58.6% conversion. This could mean that the encapsulation layer of the fumaric acid is still protecting the fumaric acid from being dissolved or the pathway is being inhibited slightly by the presence of the vegetable oil layer.

Although methane was not measured, the total potential number of [mu]moles methane that could be captured by EFA was 330 [mu]moles and 340 [mu]moles by fumaric acid.

Example 5: The Effect of Encapsulated Fumaric Acid and Fumaric Acid on the pH of Unbuffered Rumen Fluid

See above for methods. Results are shown in Figure 6.

Encapsulated fumaric acid followed the pH of the control relatively well and only caused a slight drop in pH compared to fumaric acid, which caused a drop of 0.74 pH units after only half an hour. The difference in pH was most likely due to the effect of the vegetable coating; preventing the fumaric acid dissolving immediately and slowing down the drop in pH.
Example 6: The Effect of Fumaric Acid, Bakeshure<(R)> 451 and Bakeshure<(R)> 470 on the pH and Volatile Fatty Acid Concentration of Sheep Rumen Fluid In Vivo.

See Sections above for methods. pH results are shown in Figures 6 and 7. Estimation of remaining feed is described in Figure 8. Volatile fatty acid results are illustrated in Figures 9 and 10.

Figure 6 shows little variance in pH throughout the sampling period. Statistical analyses showed no significant differences between the groups throughout the sampling period (P - value > 0.05). This is unexpected as fumaric acid caused a drop in pH in in vitro experiments, see Example 5. The lack of pH drop can however, be partially explained by the results described in Figure 8. At the end of the sampling period there was always at least 25% of the feed containing fumaric acid remaining in the feed box whereas all the other feeds were gone by the end of the day. There could be various reasons why the sheep did not eat the fumaric acid, e.g. palatability, a regulation effect or a physical effect (e.g. the fumaric acid irritated the mouth or nose). However, as the fumaric acid was not eaten completely the pH drop was not observed.

Figure 7 illustrates the pH in the rumen after the additives were added via the cannula. The decrease in pH associated with fumaric acid is clearly illustrated after half an hour post-feeding. On average, the lowest pH recorded as a result of fumaric acid was 4.40. The two Bakeshure products did not cause such a drastic drop in pH; Bakeshure<(R)> 451 reached a low of 5.78 and Bakeshure<(R)> 470 reached a low of 6.19.

Statistical analyses revealed no significant differences between the groups pre- feed (P- value = 0.810) but half an hour after feeding the difference was extremely significant (P-value = 0.000). One hour after feeding the difference between the groups was significant (P-value = 0.042) and thereafter there were no significant differences between the groups (P-value >0.05).

Although statistically, there was no significant difference between the groups (P-value > 0.05), Figure 9 shows that upon average the diet containing fumaric acid produced the lowest levels of propionic acid at each sampling time. This follows on from the estimation of remaining feed results, as the animals were not eating, lower levels of fatty acids would be expected. Both Bakeshure products were initially lower than the control but after 2 hours had risen above. The concentration of propionic acid then remained constant (approximately 18 - 20 mM) until the end of the sampling period. This seems to corroborate the theory that the Bakeshure products are broken down slowly and there is no sudden release of fumaric acid and hence no sudden conversion to propionic acid.

Statistically, there were no significant differences between the groups (P-value > 0.05).

Comparing the control and two Bakeshure products in Figures 9 and 10, the results are very similar. However, fumaric acid appears different in each graph. In Figure 10, two hours after feeding the concentration of propionic acid increased until it was higher than any other group. This could be due to the effect of pH on bacterial metabolism, as the increase in propionic acid coincides with an increase in pH, see Figure 6.

Bakeshure(R) 470 appears to produce less propionic acid than the control at least for the first two hours. This could be due to an increased delay in the release of fumaric acid compared to the Bakeshure(R) 451 release; as Bakeshure(R) 470 has a thicker layer of oil (35% to 39% compared to 13% to 17% partially hydrogenated vegetable oil respectively) it should release fumaric acid at a slower rate than Bakeshure(R) 451. Therefore, propionic acid concentration could have stayed constant for longer than the time sampled as fumaric acid was steadily released. In addition to this, there will be less fumaric acid proportionally in Bakeshure(R) 470 compared to Bakeshure(R) 451 , so less propionic acid will be produced as a consequence.

Discussion

Several compounds have been studied as potential feed additives for ruminants as a method of reducing methane production in the rumen[6], such as medium chain fatty acids[7, 8], oxaloacetate and butyrate enhancers[9], sodium fumarate[10, 11], oils[12, 13], organic acids [9, 14, 15] and fumaric acid[16]. Initial trials have shown that fumaric acid would act as an alternative hydrogen acceptor and prevent H2 reaching the methano genie archaea[17]. However, there was evidence of a decrease in rumen pH associated with addition of free acids. Therefore a method of supplying fumaric acid had to be found that decreased methane production but did not adversely affect rumen pH.

Encapsulated fumaric acid (an ingredient in tortilla flour) used in vitro was found to decrease the drop in rumen pH with no large, adverse effects upon propionic acid and methane production. Propionic acid production dropped by 4% in encapsulated fumaric acid samples compared to fumaric acid samples. Methane generation in samples containing encapsulated fumaric acid was 0.8% higher than those samples containing fumaric acid.

No drop in rumen pH was observed in sheep fed 75 g fumaric acid in 400 g Ewe Lamb feed. In addition, the two Bakeshure(R) products did not greatly affect rumen pH when fed in the diet. However, when the additives were introduced to the sheep via the cannula, fumaric acid caused a drop in rumen pH to 4.40 compared to 5.78 from Bakeshure(R) 451 and 6.19 by Bakeshure(R) 470. The difference between the two sets of data may be due to the fact that the sheep were reluctant to eat fumaric acid and often left approximately 25% of the feed at the end of the day. Palatability, a regulation effect or a physical effect (e.g. the fumaric acid irritated the mouth or nose) are all reasons why the sheep may have been reluctant to eat the acid.

An encapsulated fumaric acid product would therefore have at least two advantages over fumaric acid: -

1) Increased palatability,
2) No dangerous drop in rumen pH.
3) No increase in Na<+> uptake, causing electrolyte imbalance.

Example 7: Effect of encapsulated fumaric acid on growth, feed intake and methane production in lambs.

One hundred and twenty Welsh Mule Cross lambs (5 - 6 months old; average weight 26 kg; range 16.5 kg - 39 kg) were randomly allocated to one of the following three diets; Control (17 g partially hydrogenated vegetable oil (PHVO)/kg concentrate), fumaric acid (100 FA and 17 g PHVO/kg concentrate), EFA (117 g EF A/kg concentrate). Lambs were fed ad libitum and presented with unlimited amounts of straw and fresh water. The growth trial lasted for 56 d, during which lamb weight, feed intake and methane production were recorded. Gaseous emissions were measured using a tunnel system (Lockyer et al., 1995). The methane concentration of the sampled air was taken alternately at 4-min intervals from the air leaving or entering the tunnel and evaluated using a gas chromatograph. Data were compared by analysis of variance for live weight gain, empty body weight at slaughter and killing out percentage, blocked by pen.

Treatments had no significant effect on live weight gain (Table 6) over 56 d. However, when the first 22 d were considered, animals receiving EFA had a significantly higher rate of gain ($P<0.05$) whereas those receiving FA had a significantly lower rate of weight gain ($P<0.05$). Both FA and EFA decreased feed intake, with the effect more pronounced in the FA group. As a result of the increase in weight gain and reduction in intake, the efficiency of feed conversion was higher when EFA was added to the diet. On a numerical basis, there was a 20% boost in efficiency of the EFA supplemented animals compared to the control group.

Table 6. Daily live weight gain, concentrate intake, feed conversion, empty body weight and killing out percentage in lambs fed non-supplemented diets or diets supplemented withfumaric acid or encapsulated fumaric acid.

Control	Encapsulated Fumaric acid	SED	fumaric acid
Live weight gain days 1-43 (g/d)	182	202	168 21.6
Live weight gain days 1-22 (g/d)	205	225	161 17.9*
Live weight gain days 22-42 (g/d)	168	189	161 20.8
Intake kg/d	1.7	1.5	1.4 0.1 l<p<0 1>
Feed conversion (g gain/ kg feed intake)	108	132	119 13.7
Empty body weight at slaughter	38.7	38.6	37.3 1.45
Killing out %	50.5	50.6	50.8 0.6

Given that approximately 10% of the animals' gross energy is estimated to be lost via methane, this increase in efficiency seems rather high. However, other authors have reported that organic acids such as FA might improve rumen function (Martin, 1998) and stimulate fibrolytic activity in the rumen (Lopez et al., 1999). The empty body weight at slaughter was similar between the groups thus there was no effect of the treatments on the killing out percentage, which averaged around 50%.

Table 7. Concentrate intake and methane production in lambs fed non-supplemented diets or diets supplemented with fumaric acid or encapsulated fumaric acid.

Control	Encapsulated Fumaric acid	SED	fumaric acid
Intake (kg/d)	1.60	1.60	1.40 0.23
Methane production	23.9	6.0	12.2 1.05***

(L/d)

Both fumaric acid treatments significantly decreased methane formation (Table 7) with EFA (75% decrease in methane) being significantly ($P<0.05$) more effective than FA (50% decrease in methane). Based on a 100% conversion of fumaric acid to propionate, 150 g fumaric acid would decrease methane production by 7 L. Table 7 reveals that the actual decrease in methane (EFA, 17.9 L; FA, 11.7 L) is far greater than can be predicted stoichiometrically. The reason for this difference is unclear and requires further investigation, however it is possible that the effect of the FA is cumulative, effectively starving the ruminal methanogens of the H2 they require for methanogenesis and decreasing their numbers and activity over a period of time.

The 75% decrease in methane described in this study is the largest reported in the literature to date and as well as having an impact on the environment whereby greenhouse gas emissions are abated there are significant implications for the farming industry with increased efficiency of feed conversion.

Example 8: In Vitro Batch Fermentations

Short-term (24-hour) incubations were carried out with rumen fluid withdrawn from three rumen cannulated sheep. Rumen fluid was withdrawn, via a cannula, 2 hours after the morning feed and strained through two layers of gauze. Strained rumen fluid was taken from each sample, pooled and maintained under O2-free CO2. Rumen fluid was anaerobically transferred to the buffer described by Menke and Steingass [19] containing per litre: 475 mL distilled water, 0.12 mL trace elements solution (13.2 g CaCl2.2H2O, 10 g MnCl2.4H2O, 1 g CoCl2.6H2O and 0.8 g FeCl2.6H2O per litre), 237 mL of buffer solution (35 g NaHCO3 and 4 g (NH4)HCO3 per litre), 237 mL of main elements solution (5.7 g Na2-HPO4, 6.2 g KH2-PO4 and 0.6 g MgSO4.7H2O per litre), 1.22 mL 0.1 % (w/v) resazurin solution (100 mg/100 mL distilled water) and 47.5 mL freshly prepared reducing solution (336 mg Na2S.9H2O and 2 mL IM NaOH made up to 47.5 mL with distilled water). The final ratio of the buffer :rumen fluid was 2:1. After mixing, 50 mL of buffered rumen fluid was anaerobically dispensed to a 120 mL Wheaton bottle containing 400 mg of general purpose (GP) diet, see Appendix III, previously ground to pass through a 1 mm mesh screen. The bottles were sealed (under CO2 atmosphere) with butyl rubber stoppers and aluminium crimp caps then incubated at 39 [deg.]C for 24 hours in a Grant OLS 200 water bath.

Effect of Fumaric Acid Encapsulated in a High Melting Point Coconut Oil and Palm Oil on Methane and VFA Production

Sigma-Aldrich fumaric acid coated in coconut oil (melting point 20-28 <0>C) and palm oil (melting point 30-40 [deg.]C) was prepared described herein. 54.6 mg fumaric acid coated in either coconut oil or palm oil was added to Wheaton bottles containing 400 mg general purpose feed in triplicate. 8.2 mg of coconut oil or palm oil was added to Wheaton bottles containing 400 mg general purpose feed in triplicate. For comparison 46.4 mg fumaric acid and 54.6 mg Bakeshure 451 were also set up. Another three bottles were set up containing 400 mg general purpose feed only (controls).

After 24 hours of incubation, analyses of methane, hydrogen, carbon dioxide, nitrogen and volatile fatty acids were carried out.

Results

Different encapsulation treatments and ingredients produced different effects on methane formation from ruminal fluid in vitro (Figure 11). Statistical analysis of the results in Figure 11 showed a very significant difference (P- value 0.0018). Based on the control producing 23.17 mL methane, the following changes were observed: - Fumaric acid (FA) -11% decrease in methane production, Bakeshure 451 -19% decrease in methane production, Coconut oil -4% increase in methane production, FA + coconut oil -12% decrease in methane production, Palm oil -4% decrease in methane production, FA + palm oil -20% decrease in methane production. Bakeshure 451 decreased methane production by approximately double that of fumaric acid alone. Coconut oil alone did not decrease methane production but increased it by 4% in. Fumaric acid coated in coconut oil decreased methane by approximately the same percentage as fumaric acid alone. Fumaric acid coated in palm oil decreased methane by approximately the same percentage as the Bakeshure 451. As palm oil by itself caused only a slight decrease in methane and fumaric acid alone decreases methane by half the amount of the combined sample, it can be noted that encapsulation of fumaric acid with palm oil decreases methane production further than non-coated fumaric acid.

Volatile fatty acid production was affected in a manner consistent with effects on methane formation (Table 8).

Table 8. Effect of Various Additives on Methane and Volatile Fatty Acid Concentration.

Total Methane** C2<ns> C3*** C4* VFA*
([mu]mol/d) ([mu]mol/d) ([mu]mol/d) ([mu]mol/d) ([mu]mol/d)
Control 1034.361 892.159 357.714 324.764 1645.265
Coconut Oil 1079.070 852.847 353.627 305.461 1589.685
FA + Coconut Oil 907.596 861.591 517.752 300.305 1751.178
Palm Oil 995.093 796.534 330.282 276.559 1469.496
FA + Palm Oil 825.276 844.996 523.427 271.265 1694.909
Fumaric Acid (FA) 920.700 892.068 522.466 281.632 1752.575
Bakeshure 451 837.193 888.510 472.124 261.770 1679.950 <ns>There was no significant difference between the groups. *P<0.05; ** PO.01; **<H>
PO.001. Based on the control producing 357.7 [mu]mol/d propionate, the following changes were observed: -
Fumaric acid (FA) -46% increase in propionate production, Bakeshure 451 -32% increase in propionate production,
Coconut oil - 1 % decrease in propionate production,
FA + coconut oil -45% increase in propionate production, Palm oil -8% decrease in propionate production,
FA + palm oil -46% increase in propionate production.

Discussion

Coating fumaric acid with high melting point oils produced the same effect on methane production: methane decreased by 20%, in comparison with Bakeshure 451, which gave a 19% decrease. Coconut oil on its own did not appear to decrease methane production. This is surprising as Machm[upsilon]ller et al 1998[2O] found methane generation was decreased 43% when 26 g coconut oil/kg feed was incubated with rumen fluid. In the experiment reported here, the equivalent of 20.5 g coconut oil/kg feed was used but methane production

increased by 4%.

Fumaric acid encapsulated in either coconut oil or palm oil increased propionate concentrations to the same level as fumaric acid alone. Bakeshure 451, however, was consistently below that of fumaric acid. This shows that Bakeshure 451 is not the optimum product for decreasing methane and increasing propionate production, but by can be improved upon by changing the fat layer. The experiment reported here illustrates that other fats and different coating methods can replace or improve upon the method of preparation and chemical composition of Bakeshure. Furthermore, microscopic analysis indicated that the encapsulated fumaric acid particles used in encapsulation experiments was smaller than Bakeshure 451 particles. Thus, the size of particle appears not to be critical to the effectiveness of inhibition of methane formation from ruminal digesta in vitro. Appendices

Appendix I The Effect of Encapsulated Fumaric Acid and Fumaric Acid on the Generation Rate of Volatile Fatty Acids
Sample Time (hours)
ID 0.5<ns> 1.08<ns> 2.08<[pi]s> 3.08<ns> 4.08<ns> 5.08<ns> 22<ns> 24<ns>
Control 434.8 718.1 942.9 1151.1 1317.0 1464.9 3143.9 3356.5
Encap.FA 382.4 771.1 992.9 1136.0 1336.0 1495.4 3487.0 3515.5
FA 341.6 699.9 963.1 1113.2 1365.4 1610.3 3579.5 3664.1
Key: Encap.FA = Encapsulated fumaric acid, FA = Fumaric acid ns = Not Significant * = Significant ** = Very Significant *** = Extremely Significant

Table Al. Increase in Mean Volatile Fatty Acid Concentrations ([mu]moles/50 mL) at Various Times
Sample Time (hours)
ID 0.5*** 1.08* 2.08** 3.08* 4.08** 5.08*** 22*** 24***
Control 102.1 197.4 261.0 304.0 337.6 248.2 635.0 670 Encap.FA 87.4 231.4 309.9 352.2 402.9 306.2 895.6 904 FA 56.5 159.8 248.2 306.2 393.1 393.1 940.8 946
Key: Encap.FA = Encapsulated fumaric acid, FA = Fumaric acid ns = Not Significant * = Significant ** = Very Significant *<**> = Extreme Significant
Table A2. Increase in Mean Propionate Concentration ([mu]moles/50 mL) Over a 24 Hour Period Sample -Time (hours)
ID 0.5*** 1.08* 2.08** 3.08* 4.08** 5.08*** 22*** 24***
Control 204.2 182.8 125.5 98.7 82.7 71.6 29.0 27.9
Encap.F 174.8 214.3 149.0 114.4 98.8 87.1 41.0 37.7
A
FA 113.0 148.0 119.3 99.4 96.3 94.4 43.0 39.4
Key: Encap.FA = Encapsulated fumaric acid, FA = Fumaric acid ns = Not Significant * = Significant *<*> = Very Significant **<*> = Extremely Significant
Table A3. Mean Propionate Production Rates ([mu]moles/hour) Over a 24 Hour Period

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