Outcome of assessment of 3-Nitrooxypropanol “3-NOP” - Assessment

2.1. Section II: Identity, characterisation and conditions of use

The additive is a preparation of a minimum 10% 3-NOP (chemically synthesised), propylene glycol acting as a diluent, and precipitated and dried silicic acid acting as a carrier. The applicant provided data from eighteen batches supporting the specification values outlined below (Table 1).

Table 1: Specification table

Composition	
3-nitrooxypropanol (active substance)	Minimum 10 w/w%
Silicon dioxide	~54%
Propylene glycol	~35%
Appearance	
White, free-flowing, fine granular powder	
Chemical-physical specifications	
Purity	>98.0%
Dusting potential Particle size distribution	330 – 390 mg/m^3 Average of 290 µm; 0.4% of particles with diameter < 50µm
Bulk density	0.55 kg/L

The Group evaluated the physico-chemical and technological properties of the additive, concluding that it showed good homogeneity and that it is of low dusting potential with few small particles of respirable size.

In their first evaluation, members observed that no analysis was performed on the final product to screen for dioxins and heavy metals. A question was also raised regarding the potential degradation of the product throughout the manufacturing process, as it could not be concluded whether the additive degrades in the time between production and addition to a premixture, until its incorporation in feed, and subsequently until the feed reaches the animal. The AFFAJEG requested the applicant to provide an analysis of impurities in the final product, and clarification on the potential degradation of the additive after pelleting.

The Group raised concern over the applicant’s estimations of the additive’s stability, as the dossier claimed that approximately 10% of 3-NOP is lost during the pelleting process. The applicant was asked to clarify whether the instruction for mash preparation would include a requirement for a higher concentration of 3-NOP to compensate for this loss. Furthermore, members estimated the average loss of 3-NOP concentration 3 months after pelleting at 25.9%,

as opposed to the 17% claimed by the applicant, and requested the applicant to revisit the stability calculations and to provide information on the process by which the additive degrades.

The applicant provided a comprehensive response addressing the Group's requests. A mistake in the stability results table was corrected, consistent with the loss of 3-NOP concentration 3 months after pelleting being 17%. It was clarified that manufacturers would be advised to use a premixture containing an additional 10% of 3-NOP in pelleted feed. The AFFAJEG estimated a percentage loss of 3-NOP (15%) during and after the production process and deemed it acceptable. No impurities were detected in the analyses presented by the applicant.

The additive is intended to supply a minimum of 52.8 mg 3-NOP and a maximum of 88 mg 3-NOP per kg of complete feedstuff (moisture content of 12%) for all ruminants for milk production and reproduction. Conditions of use of the additive are summarised in Table 2:

Table 2: Conditions of use of 3-NOP as described in the application

Proposed mode of use in animal nutrition:

Additive	3-nitriixypropanol (3-NOP)
CAS number	100502-66-7
Category (ies) of additive	Zootechnical feed additive
Functional group (s) of additive	Substances that favourably affect the environment

Description:

Composition, description	Purity criteria	Method of analysis
Preparation of 3-NOP propylene glycol and silicic acid	Containing a minimum of 10% w/w, 98% pure 3-NOP	HPLC system

Trade name: Bovaer 10

Name of the holder of authorisation (if appropriate): N/A

Conditions of use:

Species or category of animal	Min max age	Min content (mg of 3-NOP per kg of complete feed with a moisture content of 12%)	Max content (mg of 3-NOP per kg of complete feed with a moisture content of 12%)	Withdrawal period
All ruminants for milk production and reproduction	From first insemination to culling	52.8mg	88 mg	-

2.1.1. Conclusions on Section II

The AFFAJEG concluded that the estimated average loss of 15% of 3-NOP from production to ingestion by the animal is acceptable, particularly given the applicant's recommendation of including a 10% overage in pelleted feeds.

No further concerns were raised for Section II of the dossiers.

2.2. Section III: Safety

A set of toxicological studies and a literature review were presented and evaluated by the Chemical Risk Assessment Unit at the FSA prior to assessment by the AFFAJEG. A list of the studies contained within the application dossier can be found in **Appendix 1.**

2.2.1. Safety for the target species

The AFFAJEG evaluated two tolerance studies presented in the application.

Study 1 aimed to find a dose range to inform Study 2 and to potentially establish a margin of safety. The applicant claimed that a margin of safety of 5 could be derived from this study, but the JEG challenged this claim based on shortcomings in its design and implementation. Study 1 used only 4 cows per group, which were given 0, 100, 500 and 1000 mg 3-NOP/kg feed DM for 90 days. The highest dose showed a reduced intake of feed and a reduced heart weight, with no pathological signs (haematology, clinical chemistry, and gross pathology at necropsy and histopathology of selected organs). It was considered that such a low sample size would be unlikely to yield reliable statistics, especially given that two cows, one from the top-dose group and one from the 500 mg group, were euthanised prematurely. Furthermore, NOPA was detected in the milk from 3 out of 4 cows from the control group. The Group did not consider this study valid for evaluating the tolerance of the target species to the additive.
Study 2 used 20 cows per group, which were given doses of 3-NOP of 0, 80, 100, or 200 mg/kg feed DM for 56 days. Statistically significant differences were found in some haematological and biochemical parameters for all dose groups. These were within normal physiological ranges and without an associated dose response, therefore, were not considered to be adverse effects. At the 200 mg dose, effects identified included decreased ovary size, decreased serum activities of ALT (alanine aminotransferase) and LDH (lactate dehydrogenase), and reduced feed and water intake. Feed and water intake vary with many factors and, since no behavioural or productivity changes (i.e., milk yield) were reported, the decrease in feed and water intake would not be considered an adverse effect. The decrease in ovarian size was not accompanied by histopathological change and it was concluded that it should not be considered an adverse effect of the study at the 200 mg/kg dose. The serum activities of LDH and ALT remained within the normal reference range and would not be considered an adverse effect. **The Group concluded that the additive could be considered safe at a dose of 200 mg/kg and that a margin of tolerance of 2 could be established.**

2.2.2. Safety for the consumer

2.2.2.1. Carcinogenicity

The applicant presented a 2-year carcinogenicity study in Wistar rats in which benign mesenchymal cell tumours were reported in 4 out of 49 females at the top dose of 300 mg/kg bw/day of 3-NOP given orally. Based on these results, the original study report concluded there was evidence of carcinogenicity in female rats. However, an independent group of pathologists reanalysed the study's slides and concluded that mesenchymal cell tumours were present in 3 out of 49 females at the top dose group, which was no longer statistically significantly different from the control group.

The AFFAJEG evaluated the data, observing that the evidence of tumour production in the medium (100 mg/kg bw/day) and low dose (50 mg/kg bw/day) female groups was inconclusive, as only one animal in each group developed mesenchymal cell tumours and this was within the historical background range of the laboratory. In addition, mesenchymal cell hyperplasia was found in two females in the top dose group (300 mg/kg/day) only. The male groups did not develop any mesenchymal tumours; however, their top dose (100 mg/kg bw/day) did produce mesenchymal cell hyperplasia. Based on this finding of mesenchymal cell hyperplasia in males at 100 mg/kg bw/day, **the NOAEL was concluded to be 50 mg/kg bw/d.**

The AFFAJEG concluded that at the higher dose levels (300 mg/kg/day in females), the additive has the potential to cause mesenchymal cell hyperplasia and benign tumours. **Due to the absence of malignant tumours and genotoxicity, it was concluded that the additive is not**

carcinogenic at the recommended inclusion rate and benign tumours occurred only above the NOAEL.

2.2.2.2. Genotoxicity

The applicant presented a package of studies to evaluate the genotoxic potential of t3-NOP. The Group evaluated the positive results found in two in vitro micronucleus assays and an equivocal result in a third in vitro micronucleus assay, which contrasted with the negative findings of the two in vivo micronucleus studies presented. It was noted that positive results occurred in Chinese hamster V79 cells, with negative results in a study using human peripheral blood lymphocytes and an equivocal result in a study using TK6 cells. Regarding the in vivo negative findings, AFFAJEG experts considered that the bone marrow would have been exposed in the study using the intraperitoneal route of exposure and the negative results of this in vivo test should be considered valid.

In the second study, using oral dosing, the results were negative except for males dosed at the top dose and sacrificed at 24 hours, in which micronuclei were statistically significantly increased compared to the negative control, but with a frequency that was within the historical control range. Based on the OECD guidance on establishing the biological relevance of a result in this assay, which is neither clearly positive nor clearly negative, AFFAJEG members recognised the requirement for external expert judgement. An external consultant, contracted by the applicant, concluded that apparent increase in micronuclei may have been an artifact due to the Giemsa-based stain that was used, to which Group experts agreed. **The Group concluded that 3-NOP is non-genotoxic in vivo.**

The JEG also evaluated the genotoxic potential of 3-NOP's metabolite 3-nitrooxy-propionic-acid (NOPA). A positive result was obtained in a bacterial reverse mutation assay, but no positive results were found in a mammalian cell in vitro micronucleus test and an in vivo gene mutation and micronucleus study in transgenic mice. **The Group concluded that the metabolite NOPA is non-genotoxic in vivo.**

2.2.2.3. ADME

The AFFAJEG evaluated the ADME data presented by the applicant. Discussions focused on the modification of the proposed ADI, the formation of 3-NOP metabolites in the rumen and the presence of NOPA in milk and edible tissues.

The Group noted that the acceptable daily intake (ADI) proposed (discussed below) was based on toxicological data for 3-NOP and evaluated whether this ADI could also be applied to its metabolite NOPA. The application presented ADME studies performed in rats, which showed that NOPA is the primary metabolite of 3-NOP, and ADME studies in ruminants, which demonstrated that 3-NOP is rapidly metabolised to NOPA. Levels of NOPA in the plasma of cattle also decline quickly over a period of three hours. **The Group concluded, that, given the extent of metabolism of 3-NOP to NOPA in rats, an ADI established based on toxicological data for 3-NOP could also be applied to its metabolite NOPA.**

Oxetane, a potential alkylating agent, was identified as a metabolite of 3-NOP in an in vitro study to investigate the metabolism of 3-NOP in goat, sheep and cow rumen fluids in the presence of feed under anaerobic conditions. However, the AFFAJEG noted that oxetane was not found in the in vivo studies and that it could be an artefact present in the in vitro study, rather than a metabolite of 3-NOP. **AFFAJEG experts noted that oxetane was very unlikely to persist within the rumen**, as it would be metabolised rapidly, with minimal release to the small intestine and negligible impact on the rumen.

From in vitro studies using rumen fluids, the main metabolite of 3-NOP in the rumen is 1,3 – propanediol, which is not expected to accumulate. **The Group concluded that propanediol would not be a cause for concern in the target species.** Members also noted that, in plasma, the main metabolite of 3-NOP was 3-nitrooxypropionic acid (NOPA), with other metabolites also subsequently formed, such as 3-hydroxypropionic acid within the first 24 hours.

2.2.2.4. ADI

The Group evaluated the modification by the applicant of the proposed acceptable daily intake (ADI) of 3-NOP, from 1 to 0.3 mg/kg bw/d, based on the recommendation of the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment in May 2019. The COT had been asked to advise on male reproductive toxicity observed in the chronic oral toxicity study and shorter-term studies in rats. Due to the severity of effects on the male reproductive system and the steepness of the dose-response relationship, the COT advised that an uncertainty factor of 300 should be applied, rather than the standard 100, and that the relevant point of departure for the male reproductive effects was the BMDL5 of 95.6 mg/kg bw/day for decreased testicular weight. This proposed ADI also provided a margin of exposure of 167 to the NOAEL for mesenchymal cell hyperplasia in males observed in the carcinogenicity study. **The AFFAJEG concluded that for 3-NOP an ADI of 0.3 mg/kg bw can be considered appropriate.**

2.2.2.5. Exposure assessment and risk characterisation

The JEG evaluated the residue data presented in the application to determine the presence of 3-NOP and NOPA in milk and edible tissues. The applicant evaluated the exposure to NOPA from milk using the lower limit of quantification (LLOQ) level of 5 µg/kg used in the tolerance and efficacy studies presented, which showed no detectable concentrations of NOPA from all milk sampled. Toxicologist experts of the JEG carried out a conservative exposure assessment based on the JECFA food basket approach, showing that the estimate of exposure to NOPA at the LLOQ was lower than the ADI of 0.3 mg/kg bw/d established for 3-NOP by two orders of magnitude. The AFFAJEG took account of the results of all of the toxicological studies listed in Appendix 1 in deciding on the value for the ADI for 3-NOP. **The AFFAJEG compared their exposure assessment results to those presented by the applicant and concluded that, based upon the LLOQ concentration of 5 µg/kg, the levels of NOPA residues in milk were low enough not to be cause for concern.**

2.2.3. Safety for the user

The applicant presented a comprehensive set of data to support evidence of safety for the user. It was noted that the studies were carried out using the active substance (3-NOP) itself, as opposed to the final formulated product but this was not considered to be a cause for concern and tests were considered representative of the product. The studies included in the application were:

- ACute inhalation toxicity
- Local lymph node assay for skin sensitisation
- bovine corneal opacity and permeability test for eye irritancy
- In vitro skin corrosion and irritation tests

Based on the data presented, the AFFAJEG concluded that the additive should be considered corrosive to the eyes and a skin irritant but not corrosive to skin or a sensitiser to skin. The applicant claimed that the additive should not be considered as harmful by inhalation, but AFFAJEG experts noted that some adverse effects were found in the acute inhalation study presented and recommended that measures to control exposure, such as masks, may need to be considered when handling the additive.

2.2.4 Safety for the environment

The AFFAJEG evaluated the environmental risk assessment carried out by the independent expert Dr. Chris Sinclair, a member of the Register of Specialists of the FSA. A detailed assessment was carried out for Phases I and II-A. The environmental risk assessment provided by the applicant was noted to have significant deviations from the expected approach, some through error but mainly due to the specific properties and behaviour of 3-NOP. However, the environmental risk assessment studies that were provided were deemed acceptable to evaluate the safety for the environment of the additive.

The environmental risk assessment evaluation of 3-NOP could stop at question 3 of the Phase I risk assessment as set out in the relevant guidance3, since there is clear evidence that in dairy cows it is extensively metabolised to a range of endogenous compounds resulting in minimal environmental exposure**. No further assessment of the environmental risks of 3-NOP was considered necessary and it was concluded that the use of 3-NOP poses an acceptable risk to the environment.**

2.2.5 Conclusions on safety

- the AFFAJEG concluded that the additive can be considered safe for the target species at a maximum dose of 200 mg/kg DM (176 mg/kg in complete feed), establishing a margin of tolerance of 2 from the intended concentration of use.
- an ADI of 0.3 mg/kg bw/d was established for 3-NOP and NOPA.
- metabolism of 3-NOP produces 1,3 - propanediol in the rumen. Propanediol does not accumulate in the rumen and is no cause for concern.
- levels of 3-NOP or its primary metabolite NOPA in milk and edible tissues were not deemed as being of concern as consumer intakes would be well within the ADI.
- the additive should be considered corrosive to the eyes, a skin irritant and potentially harmful by inhalation.
- the additive poses an acceptable risk to the environment.

2.3 Section IV: Efficacy

The Group evaluated Section IV of the dossier, containing evidence of efficacy, presented in three distinct sections: three in vitro studies, two meta-analyses and three long-term dairy cow efficacy trials. The rapporteurs presented the information to the group.

2.3.1 In-Vitro studies

In-vitro study 1 was a straight dose-response study using doses of 0, 5, 10 and 20 mg/day of 3-NOP, showing no significant differences in response. In-vitro study 2 used a dose of 2 mg/day of 3-NOP. In-vitro study 3, used a dose of 500 mg/kg of DM, which was later adjusted to the recommended dose proposed in the application.

The Group noted that in all studies, regardless of the inclusion level of 3-NOP, methane production was significantly reduced. Members also discussed shortcomings in the in-vitro studies. Study 1 included a level of crude protein of 10.5%, which is not representative of diets for dairy cows. Study 3 presented a very high level of crude protein (23.6%), not very representative of a typical UK cattle diet and different from the control diet (17.9%). It was also noted that none of the studies used a grass, or grass silage-based, diet representative of a large proportion of dairy diets in the UK. A ruminal volatile fatty acid (VFA) reduction was observed in studies 1 and 3, as well as an increase in hydrogen levels in all three studies. The authors noted that excess hydrogen would be eructed, contributing to an energy loss. Results from studies 1, 2 and 3 can be found in Table 3, 4 and 5, respectively:

Table 3: Study 1. Effect of 3-NOP on the gas production in vitro

Gas production	Treatment (3-NOP mg) 0	Treatment (3-NOP mg) 5	Treatment (3-NOP mg) 10	Treatment (3-NOP mg) 20	P-value (b) Trt	P-value (b) Lin	P-value (b) Quad
Total (L/d)	1.12	1.07	1.14	1.14	0.71	0.56	0.8
CH_4 (mL/d)	27.8a	6.7b	4.3b	4.0b	<0.01	<0.01	<0.01
CH_4 (mL/g DM)	2.82a	0.7b	0.44b	0.39b	<0.01	<0.01	<0.01
CH_4 (mL/g DMD)	4.93a	1.25b	0.78b	0.69b	<0.01	<0.01	<0.01
H_2 mL/d)	13.1a	33.9b	40.0b	41.6b	<0.01	<0.01	<0.01

a, b within a row, means without a common letter differ significantly. Trt = Treatment effect; Lin = linear effect; Quad = quadratic effect

Table 4: Study 2 Effect of 3-NOP on the gas production in vitro

Gas production	Treatment period: Control	Treatment period 3-NOP (2 mg)	Treatment period P-value	Recovery period: Control	Recovery period: 3-NOP (2mg)	Recovery period: P-value
Total (L/d)	1.27	1.10	0.03	1.14	0.98	0.06
CH_4 (mL/d)	36.5	10.4	<0.01	26.1	15.2	<0.01
CH_4 (mL/g OMD)	7.79	2.32	<0.01	-	-	-
H_2 mL/d)	22.9	38.1	<0.01	13.1	16.2	0.53
CH_4 (%)	2.85	0.98	<0.01	2.61	1.15	<0.01

OMD: Organic matter digested

Table 5: Study 3 Effect of 3-NOP on the gas production in vitro

Measurement: Gas production (l/d)	Substrate: Control	Substrate: 3-NOP	SEM	p-value^
Total	0.74	0.63	0.066	0.083
Total GHG (CO_2-eq) C	1.00a	0.34b	0.122	<0.001

Measurement: GHG (% Total)	Substrate: Control	Substrate: 3-NOP	SEM	p-value^
Methane	17.1a	5.0c	0.84	<0.001
Hydrogen	2.0c	10.3a	0.99	<0.001
Carbon dioxide	80.9c	84.7b	1.19	<0.001
Nitrous oxide	0.00b	0.00b	0.006	-
$_{d}H_2$ E	40.8b	53.7a	3.79	<0.001

A: within a row, means with different superscripts differ ($p<0.05$)
B: Average of data collected in all vessels during 6 consecutive days (day 8-13)
C: sum of CH4, H2, CO2, and N2O produced corrected for their 100-year global warming potential (GWP) (CO2: 1, CH4: 28, N2O : 265, H2: 5.6
D: Gas percentages are based on the sum of H4, H2, CO2, and N2O produced
E: Average of data collected in 4 vessels/day during 4 consecutive days (day 8-11)

2.3.2. Short term efficacy studies

Two meta-analyses were presented to account for short-term efficacy studies.

The first meta-analysis used twelve in vivo studies from ten scientific publications covering dairy cows, beef cattle and sheep, with 3-NOP doses ranging from 0 to 180 mg/kg DM. The AFFAJEG judged that the first meta-analysis should not be considered for the evaluation of efficacy, given the insufficient statistical detail presented.

The second meta-analysis evaluated data from 11 experiments and 38 treatments from 9 different studies performed in beef and dairy cattle. Members noted the positive correlation between the dose of 3-NOP and the reduction of methane. However, a negative correlation was detected with dietary neutral detergent fibre content, where the higher the NDF level, the lower the effect of 3-NOP observed in methane reduction. The Group highlighted that for 3-NOP to reduce methane concentration, it would have to be fed daily to the target animals. A summary of the data identified in the second meta-analysis can be found in Table 6:

Table 6: Meta-analysis 2. Descriptive statistics of feed intake, dietary characteristics, and CH4 emission

Characteristic	Dairy cattle: Mean	Dairy cattle: Median	Dairy cattle: SD	Dairy cattle: Minimum	Dairy cattle: Maximum
DMI (kg / d)	22.3	19.35	4.13	18.3	28.0
Roughage (% of DM)	55	60	7.7	38	61
NDF (g / kg DM)	319	309	52.2	265	398
CP (g / kg DM)	178	182	15.3	161	196
BW (kg)	632	664	44	573	673
3-NOP dose (mg / kg DM)	81	68	41.2	27	135
CH_4 (g / d)	351	368	94.1	132	487
MD CH_4 (g / d)	-126	-147	664.7	-240	-27
Relative MD CH_4 (% control)	-29.6	-30.8	16.89	-64.5	-6.4
CH_4 yield (g / kg DMI)	16.1	16.3	4.61	7.2	22.4
MD CH_4 yield (g / kg DMI)	-5.2	5.0	2.94	-10.6	-1.0
Relative MD CH_4 yield (% control)	-28.1	-29.1	16.41	-59.6	-4.8

MD (mean difference) is 3-NOP group mean - control group mean

2.3.3. Long term efficacy studies

Three long-term studies were evaluated by the AFFAJEG. The three studies shared a similar experimental design and were carried out over 19 weeks, with a target dose of 60 mg 3-NOP/ kg DM in the partial mixed ration for Holstein Friesian dairy cattle. Study 1 used 64 animals, study 2 used 42 and study 3 used 48 animals. Treatment and control groups were distributed evenly. The JEG concluded that the three studies showed similar results, with an effective reduction of methane of 21% to 33%, and that they were carried out to a high standard, with sufficient sample size and a study design that treated and fed all animals individually, where each animal can be considered an experimental unit. A summary of the results from the three long-term efficacy trials can be found in Table 7:

Table 7: CH4 emission (g/d) of treatment groups in the three studies

Covariate Period

-	Study 1: No. cows	Study 1: Mean	Study 1: StD	Study 1: P-value	Study 2: No. cows	Study 2: Mean	Study 2: StD	Study 2: p-value	Study 3: No. cows	Study 3: Mean	Study 3: StD	Study 3: p-value
Control	31	464.40	54.204	-	20	406.08	48.999	-	24	368.94	43.624	-
3-NOP	32	480.43	56.110	-	20	400.19	53.013	-	24	402.61	63.202	-

Trial period

Week 1 to 3

-	Study 1: No. cows	Study 1: Mean	Study 1: StD	Study 1: P-value	Study 2: No. cows	Study 2: Mean	Study 2: StD	Study 2: p-value	Study 3: No. cows	Study 3: Mean	Study 3: StD	Study 3: p-value
Control	31	433.76	42.170	-	20	442.60	55.894	-	24	388.65	45.873	-
3-NOP	32	356.87	41.303	****	20	266.50	38.421	****	24	296.73	40.706	****

Week 4 to 6

-	Study 1: No. cows	Study 1: Mean	Study 1: StD	Study 1: P-value	Study 2: No. cows	Study 2: Mean	Study 2: StD	Study 2: p-value	Study 3: No. cows	Study 3: Mean	Study 3: StD	Study 3: p-value
Control	31	415.23	38.752	-	20	445.45	46.903	-	24	404.50	50.813	-
3-NOP	32	340.89	41.450	****	20	287.78	34.160	****	24	312.70	46.544	****

Week 7 to 9

-	Study 1: No. cows	Study 1: Mean	Study 1: StD	Study 1: P-value	Study 2: No. cows	Study 2: Mean	Study 2: StD	Study 2: p-value	Study 3: No. cows	Study 3: Mean	Study 3: StD	Study 3: p-value
Control	31	433.79	42.255	-	20	445.17	43.648	-	23	414.22	41.111	-
3-NOP	32	339.18	40.595	****	20	316.82	29.948	****	24	325.66	43.684	****

Week 10 to 12

-	Study 1: No. cows	Study 1: Mean	Study 1: StD	Study 1: P-value	Study 2: No. cows	Study 2: Mean	Study 2: StD	Study 2: p-value	Study 3: No. cows	Study 3: Mean	Study 3: StD	Study 3: p-value
Control	27	413.22	40.196	-	20	460.93	43.179	-	23	389.06	43.134	-
3-NOP	31	332.93	44.549	****	20	285.35	33.814	****	24	319.93	54.181	****

Week 13 to 15

-	Study 1: No. cows	Study 1: Mean	Study 1: StD	Study 1: P-value	Study 2: No. cows	Study 2: Mean	Study 2: StD	Study 2: p-value	Study 3: No. cows	Study 3: Mean	Study 3: StD	Study 3: p-value
Control	27	400.18	40.859	-	19	456.91	37.122	-	23	412.86	46.368	-
3-NOP	31	313.06	40.338	****	20	297.02	30.259	****	24	328.13	43.807	****

Total Period: Study 1

-	Nb cows	LS Mean	StdE	p-value
Control	31	423.24	4.375	-
3-NOP	32	331.62	4.297	****

Diff (%): -21.6

Total Period: Study 2

-	Nb cows	LS Mean	StdE	p-value
Control	20	449.13	5.741	-
3-NOP	20	292.05	5.735	****

Diff (%): -35

Total Period: Study 3

-	Nb cows	LS Mean	StdE	p-value
Control	24	410.35	5.696	-
3-NOP	24	308.11	5.639	****

Diff (%): -24.9

StD: Standard Deviation / StE: Standard Error / ****: p<0.0001

The AFFAJEG concluded that 3-NOP is efficacious at reducing methane excretion in ruminants at the proposed dose. No negative effect in animal production was observed.

2.3.4. Conclusions on efficacy

The AFFAJEG concluded that the product can be considered efficacious for reducing methane production in ruminants when used on a daily basis at the proposed dose.

The AFFAJEG noted that a theoretically predicted increase in ruminal propionate and ruminal energy efficiency arising from excess hydrogen was not demonstrated.