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Safety and efficacy of a feed additive consisting of guanidinoacetic acid for all animal species (Alzchem Trostberg GmbH)

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of guanidinoacetic acid (GAA) when used as nutritional additive in feed and water for drinking for all animal species. The FEEDAP Panel concludes that GAA at 1,200 mg/kg complete feed is safe for chickens for fattening, piglets and pigs for fattening. This concentration in complete feed would correspond to maximum concentrations in water of 600 mg GAA/L for chickens for fattening, piglets and pigs for fattening. The Panel is not in a position to conclude on a safe level of GAA in laying/reproductive birds. In the absence of data on ruminants and salmonids, the FEEDAP Panel cannot conclude on the safety of GAA for all animal species. There is no concern on consumer safety resulting from the use of GAA in feed for poultry and pigs at the proposed conditions of use. The limited data do not allow to conclude on the safety for the consumer when the additive is used in feed for ruminants or fish. GAA is not toxic by inhalation, it is not an irritant to skin and eyes, and it is not a dermal sensitiser. The FEEDAP Panel concludes that the use of GAA as feed additive is not expected to pose a risk to the environment. The use of the additive under assessment in animal nutrition at the proposed conditions of use has the potential to be efficacious in all growing avian, Suidae and ruminant (except for preruminants) species; in growing fin fish other than salmonids and in frog. It is not possible to conclude on the efficacy of the additive in other species, and in reproductive animals.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003¹ establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Alzchem Trostberg GmbH² for authorisation of the product guanidinoacetic acid when used as a feed additive for all animal species (category nutritional additives, functional group amino acids, their salts and analogues).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 28 September 2020.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product guanidinoacetic acid, when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of EFSA issued two opinions on the safety and efficacy of guanidinoacetic acid for chickens for fattening and for pigs (EFSA, 2009; EFSA FEEDAP Panel, 2016).

GAA is authorised (3c372) as nutritional additive for chickens for fattening, weaned piglets and pigs for fattening.³

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier⁴ in support of the authorisation request for the use of guanidinoacetic acid as a feed additive.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

The European Union Reference Laboratory (EURL) considered that the conclusions and recommendations reached in the previous assessment regarding the methods used for the control of guanidinoacetic acid in animal feed are valid and applicable for the current application.⁵

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of guanidinoacetic acid is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on the assessment

¹ Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

² Alzchem Trostberg GmbH. Dr. Albert Frank Str. 32, 83308 Trostberg, Germany.

³ Commission implementing regulation (EU) 2016/1768 of 4 October 2016 concerning the authorisation of guanidinoacetic acid as a feed additive for chickens for fattening, weaned piglets and pigs for fattening and repealing Commission Regulation (EC) No 904/2009. L 270/4, OJ 5.10.2016, 3 pp.

⁴ FEED dossier reference: FAD-2020-0050.

⁵ The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2011-0043-GAA.doc_.pdf

of the efficacy of feed additives (EFSA FEEDAP Panel, 2018), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The subject of this assessment is guanidinoacetic acid (GAA) when used as a nutritional feed additive (functional group: amino acids, their salts and analogues) for all animal species. The additive is currently authorised for its use in chickens for fattening, weaned piglets and pigs for fattening, and the current application is for the extension of its use in feed for all animal species.

3.1. Characterisation

The manufacturing process and the characterisation of the active substance were already described in a previous opinion (EFSA FEEDAP Panel, 2016).

3.1.1. Characterisation of the guanidinoacetic acid crystalline powder

The product is currently authorised as a powder with a minimum content of 98% GAA (on DM basis) and a maximum of 0.5% dicyandiamide and 0.03% cyanamide. The applicant requests to keep the same specifications.

The analysis of five batches (ion chromatography) showed an average of 98.7% of GAA as is (range 98.3–99.2%) and moisture ranged < 0.1–0.2%. Other components analysed were glycine that averaged 2,500 mg/kg (range 1,700–3,200 mg/kg), dicyandiamide 760 mg/kg (range 500–1,000 mg/kg), melamine 3.7 mg/kg (range 2.1–4.7 mg/kg). Cyanamide was found below the limit of quantification (LOQ, 7 mg/kg) in all five batches.⁶ Ammeline, ammelide and cyanuric acid were analysed in five batches. On average, ammeline content was 1.3 mg/kg (range 0.8–1.9 mg/kg), ammelide was 0.14 mg/kg (range 0.06–0.31 mg/kg) and cyanuric acid was < LOQ (0.05 mg/kg) in all batches.⁶

The level of cadmium, lead, mercury and arsenic was analysed in three batches of GAA, all showing values below the respective LOQ.⁷

The detected amount of these impurities does not represent safety concerns.

GAA is a white crystalline powder having a solubility in water of 1.5 g/L at 15°C and of 5.5 g/L at 20°C. Bulk density measured in three batches ranged from 340 to 370 kg/m³ while tapped density ranged from 560 to 590 kg/m³.

The dusting potential was analysed (Stauber-Heubach method) in three batches of the GAA crystalline and it ranged from 3.8 to 7.5 g/m³.⁸ Particle size distribution of GAA was measured in six batches by laser diffraction and the fractions of particles with a diameter < 11, < 50 and < 105 µm were 40–44%, 93–94% and 100%, respectively.⁹

3.1.2. Characterisation of the formulated additive

The additive should also be placed on the market in a formulated form (Creamino[®]), which is specified to contain ≥ 96% GAA, ≤ 1% water and ~ 1% starch.

The specification was confirmed by the analysis of five batches, showing a mean of 98.0% GAA (range 97.4–98.7%) and 0.2% moisture (range < 0.1–0.3%).¹⁰ Melamine content was on average 4.4 mg/kg (range 3–6 mg/kg).

The formulated additive is an off-white granular product. Its solubility in water is 5.53 g/L at 20°C.¹¹ It has a bulk density (three batches) ranging from 605 to 610 kg/m³ and a tapped density (three batches) ranging from 690 to 694 kg/m³.¹²

The dusting potential of the formulated additive was analysed in three batches (Stauber-Heubach) and ranged from 5.12 to 6.15 g/m³.¹² The particle size distribution measured by sieving of 15 batches

⁶ Technical dossier/Supplementary information August 2021/Annex II.2.1.3.1 updated.

⁷ Technical dossier/Supplementary information August 2021/Annex II.2.1.3.1 updated. LOQ in mg/kg was 0.02 for cadmium and arsenic, 0.04 for mercury and 0.32 for lead.

⁸ Technical dossier/Section II/Annex II.2.1.5.7.

⁹ Technical dossier/Section II/Annex II.2.1.5.5.

¹⁰ Technical dossier/Supplementary information August 2021/Annex II.2.1.3.3 updated.

¹¹ Technical dossier/Section II/Annex II.2.1.5.8.

¹² Technical dossier/Section II/Annex II.2.1.5.6.

showed that the fraction of particles having a diameter < 63 µm was on average 3% and the fraction < 100 µm was on average 4%. The mean size was on average 478 µm.¹³

3.1.3. Stability and homogeneity

The applicant submitted new studies on the shelf-life, stability in feedingstuffs performed with the formulated additive; and stability in water performed with the GAA crystalline powder which complement those already assessed in the previous opinion (EFSA FEEDAP Panel, 2016).

The shelf-life of the formulated additive was tested when stored in plastic bags at 25°C for 5 years (six batches) or at 40°C for 1 year (three batches). At the end of the storage period, a loss up to 1% was observed in half of the batches stored at 25°C and no losses were observed in the batches stored at 40°C.¹⁴

The stability of the formulated additive (one batch, containing 97.6% GAA) at different supplementation levels (600 and 1,200 mg/kg) in two complete pelleted feeds (starter and grower, based on maize and soybean meal) for chickens for fattening was studied.¹⁵ After mixing, the feeds were conditioned and pelleted (at 75°C for the starter and 72°C for the grower feed). Feed processing caused no losses of GAA. The samples were packed in paper bags and stored either at 25°C and 60% RH for 48 months or at 40°C and 75% RH for 6 months. As regards the starter feed, it showed losses of GAA ranging from 0% to 3% after storage at 25°C and a loss of 3% after storage at 40°C. The grower feed showed no loss of GAA after storage at 25°C, and losses were between 1% and 3% when stored at 40°C.

The stability of one batch of the formulated additive was tested in two mash feeds (starter and grower) for chickens for fattening consisting of maize, soybean meal, DDGS and maize gluten meal (containing ~ 2,600 mg/kg choline chloride), when supplemented at 600, 1,200 and 1,800 mg/kg feed.¹⁶ The samples were packed in paper bags with plastic liners and stored either at 25°C for 12 months or at 40°C for 6 months. As regards the starter feed, losses ranged from 0 to 5% when stored at 25°C and from 1% to 8% when stored at 40°C. The grower feed showed losses ranging from 2% to 10% when stored at 25°C and from 0% to 11% when stored at 40°C.

The stability of GAA crystalline powder (three batches) was tested in water at different pH (4, 7 and 9 buffered solutions and in not buffered water) and concentrations (200 and 600 mg/L, two replicates per concentration) when stored at 25°C for 4 days.¹⁷ The solutions were tightly capped during storage. No losses were observed at buffered solutions of pH 4 and 7 or in the non-buffered water. A loss of about 1% was observed in the buffered solution of pH 9 at the end of the storage period.

The homogeneous distribution of the additive in feed was studied in starter and grower feeds for chickens for fattening, in mash and in pelleted form when supplemented at 600 or 1,200 mg of formulated additive/kg.¹⁸ Ten subsamples of each feed were analysed, and the coefficient of variation ranged from 8% to 12% in mash feeds and from 2% to 4% in pelleted feeds without substantial differences attributable to the supplementation level.

3.1.4. Conditions of use

GAA is intended to be used in feed (directly or via premixtures) for all animal species at a minimum content of 600 mg GAA/kg complete feed and a maximum content of 1,200 mg/kg complete feed.¹⁹ GAA is also intended to be used in water for drinking for poultry and pigs,²⁰ at a minimum content of 200 mg/L and a maximum content of 600 mg/L.

3.2. Safety

To support the safety of the additive, the applicant performed an extensive literature search in three databases (CAB abstracts, PubMed and Veterinary Science Database) including publications from

¹³ Technical dossier/Section II/Annex II.2.1.5.1.

¹⁴ Technical dossier/Section II/Annex II.2.4.1.3.

¹⁵ Technical dossier/Section II/Annex II.2.4.1.7.

¹⁶ Technical dossier/Section II/Annexes II.2.4.1.8 and II.2.4.1.9.

¹⁷ Technical dossier/Section II/Annex II.2.4.1.11.

¹⁸ Technical dossier/Section II/Annex II.2.4.1.6.

¹⁹ Standardised feed with 88% dry matter.

²⁰ Technical dossier/Supplementary information August 2021/0_Creamino_cover_letter.

1990 onwards without language restriction.²¹ The search strategy is described in the dossier and included terms related to the active substance and the safety and efficacy for target animals and safety for humans. Clear and logical inclusion and exclusion criteria were established.

3.2.1. Absorption, distribution, metabolism and excretion

The metabolic fate of GAA was described in previous opinions (EFSA, 2009; EFSA FEEDAP Panel, 2016). In summary, GAA is an endogenous compound of the biosynthetic pathway of creatine, common to all vertebrates. It is synthesised from the amino acids glycine and arginine, mainly in the kidney and pancreas. This first step was found to be rate-limiting as the enzyme L-arginine-glycine amidinotransferase (AGAT) is subject to feedback inhibition by creatine. The second step occurs in the liver where the enzyme GAA N-methyltransferase (GAMT) catalyses the transfer of a methyl group from S-adenosylmethionine (SAM) to GAA to form creatine and S-adenosylhomocysteine (SAH). No reverse transformation of creatine to GAA occurs. Creatine is released from the liver into the circulation where it can be taken up, via a specific transporter, by various tissues. Creatine, besides its energy-related functions, undergoes an irreversible non-enzymatic conversion to creatinine which is excreted in the urine. Daily turnover of creatine must be replaced from dietary animal protein or de novo synthesis from GAA. SAH formed during this process is subsequently hydrolysed, thus generating homocysteine.

Some of the scientific papers reviewed from the extensive literature review provided new information on ADME of guanidinoacetic acid and are described below.

Tossenberger et al. (2016) showed in colostomised chickens that the apparent faecal digestibility of GAA was 99% and 98% for GAA levels of 600 and 6,000 mg/kg complete feed, respectively (excreted at very low levels in the faeces). The major excretion occurred in the urine principally as unchanged GAA followed by creatine and creatinine. Considering the renal excretion of GAA and creatine/creatinine, the true availability of GAA was estimated to be 76% at 600 mg/kg and 46% at 6,000 mg/kg feed.

In ruminants (cattle), about 50% of the GAA infused in the rumen (infusion up to 20 g/day) is degraded (Speer et al., 2020). The other half reaches the abomasum and further the intestinal tract, resulting in increased plasmatic creatine and derived metabolites (Ardalan et al., 2015), the same as in other species, e.g. the chicken (see above) or humans (Edison et al., 2013). Li et al. (2020) observed that the dietary supplementation of Angus bulls with up to 900 mg GAA/kg DM for 104 days resulted in increased cellobiase, pectinase and protease enzymatic activities in rumen, and in propionate production and microbial protein synthesis (derived from lower ammonia-N) in the rumen. These results suggested that dietary GAA may be used by rumen microbes to support their growth and modulating the composition of the microbiota. Ardalan et al. (2020) showed that abomasal infusion of GAA (10, 20, 30 and 40 g /day,²² with or without supplemental methionine) in heifers resulted in increases of plasma creatine and the requirement for methyl donors (as evidenced by the results at the two highest GAA infusion levels without concomitant infusion of methionine).

3.2.2. Toxicological studies

Repeated dose oral toxicity studies were already evaluated in a previous opinion (EFSA, 2009) where it was concluded that 'The effects reported in the 28- and 90-day studies generally reflect physiological responses to high exposures to a metabolic intermediate and do not identify any novel or unexpected toxicity'. No new evidence that would change the conclusions previously reached was provided by the applicant.

The genotoxicity potential of the additive was previously evaluated (EFSA, 2009; EFSA FEEDAP Panel, 2016) based on the assessment of an Ames test, an in vitro mammalian cell gene mutation test, and an in vitro chromosomal aberration assay. Overall, the FEEDAP Panel concluded that there was no evidence of mutagenic activity, and that the chromosomal aberration test did not show a potential for genotoxicity of the additive under assessment.

To evaluate the potential of GAA (purity 99.2%) to induce chromosome damage (aneugenicity and clastogenicity), an in vitro micronucleus test was performed in whole blood human lymphocytes according to OECD Test Guideline (TG) 487 (2016) and following good laboratory practice (GLP).²³

²¹ Technical dossier/Section III/Subsection 3.1.1.

²² The estimated amount of GAA in feed to reach 10 g GAA/day in the abomasum (considering a loss of 50% GAA due to ruminal degradation) is similar to 2,500 mg GAA/kg DM feed.

²³ Technical dossier/Supplementary information August 2021/Annex III.3.2.2.2.

Based on the results of a preliminary cytotoxicity test, the compound was tested up to 10 mM applying a short treatment (4 + 40 h of recovery) in the absence and presence of metabolic activation and a continuous treatment (44 + 0 h of recovery) without metabolic activation. Exposure started 48 h after culture initiation and the cells were cultured for a total time of 92 h. The highest concentration tested corresponded to the top dose recommended by OECD TG 487. Appropriate positive and negative control chemicals were used, and the results obtained confirmed that the experimental system was sensitive and valid. Cytotoxicity up to 35% relative to vehicle control was observed at 10 mM after short treatment with metabolic activation. The analysis of micronuclei was performed in binucleated cells blocked by 6 µg/mL cytochalasin B. No significant increase in the frequency of micronucleated cells was induced by GAA at any tested concentration. Based on these results, the Panel concluded that GAA did not induce structural and numerical chromosomal damage under the experimental conditions applied in this study.

3.2.3. Safety for the target species

The FEEDAP Panel, on the basis of tolerance studies in chickens for fattening and weaned piglets, concluded in its previous opinion that a concentration of 1,200 mg GAA/kg complete feed could be considered safe for chickens for fattening, weaned piglets and pigs for fattening, given that an adequate dietary supply of methyl donors is provided (EFSA FEEDAP Panel, 2016). In the absence of adequate data, the FEEDAP Panel could not conclude on the safety of GAA for breeder hens and roosters.

To support the safety of the additive for all animal species, the applicant provided a tolerance study in laying hens and conducted an extensive literature search (described at the start of Section 3.2). From a total of 885 references retrieved, 73 were considered relevant after screening title and abstract, and subsequently reduced to 20 after full-text revision.²⁴ From these 20, 19 references were not further considered because (i) they were studies already considered in the previous opinion (EFSA FEEDAP Panel 2016), (ii) the studies did not include relevant endpoints for the safety assessment or (iii) there were some deficiencies in the study design or execution. Therefore, only one publication was considered relevant by the Panel.

The literature reviewed did not provide new information on the safety of the additive for chickens for fattening or pigs.

3.2.3.1. Laying hens

The applicant submitted a new tolerance study in laying hens.²⁵ A total of 390 Lohmann Brown classic laying hens of 25 weeks of age were randomly distributed in 30 floor pens and the pens allocated to one of the five dietary treatments (six replicates/treatment). The experimental diet, based on maize and soybean meal and containing < 20 mg creatine/kg, 4 g methionine/kg, 1,200 mg choline/kg, 0.9 mg folic acid/kg and 17 µg vitamin B₁₂/kg, was either not supplemented (control) or supplemented with the formulated additive at 1,200 (1× maximum use level), 3,600 (3×), 6,000 (5×) or 12,000 (10×) mg GAA/kg feed (confirmed by analysis) and fed for 64 days. Body weight of the hens was determined in 4-week period (pen basis). Feed intake was determined weekly per pen. Pen laying rate and egg mass were recorded daily. Individual egg weight and egg quality parameters (egg yolk colour, weight and shell index of dried shells) were determined on days 28 and 56. Two eggs per pen (12/treatment) were randomly collected on day 57, freeze-dried (whole egg) and analysed for GAA, creatine, creatinine and homocysteine contents. Two hens per pen (12/treatment) were killed on day 62 and subjected to necropsy, and two other hens per pen were sampled on day 64 for blood analysis (for haematology²⁶ and clinical chemistry,²⁷ including GAA, creatine, creatinine and homocysteine). Data were statistically analysed by ANOVA using the pen as a statistical unit for performance data and the individual animal (or egg) for blood parameters, necropsy data and metabolites in eggs. Differences between groups were tested with Tukey test and the significance was set at $p < 0.05$.

²⁴ Technical dossier/Section III.3.1.1.1.

²⁵ Technical dossier/Supplementary information August 2021/Annex III.3.2.1.2.3.

²⁶ Haematological analyses consisted of determination of erythrocytes, haemoglobin, haematocrit, mean cell volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), heterophils, monocytes, lymphocytes, thrombocytes, T-cell and B-cell counts.

²⁷ Clinical chemistry consisted of plasmatic determination of aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (AP), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), total protein, albumin, cholesterol, triglycerides, bile acids, urea, uric acid, sodium, potassium, chloride, calcium, magnesium and phosphorous.

Mortality was low (two birds in the 5× group and one in the 10× group) and not treatment related. Hens' body weight at the end of the study was significantly lower in the groups treated with 6,000 and 12,000 mg GAA/kg compared to the control (1,858 and 1,882 g vs 2,011 g, respectively). Daily feed intake was significantly reduced in all treatment groups (121, 119, 120 and 113 g/d vs 130 g/d in the control group) and there was no improvement in feed to egg mass ratio (2.12 in the control group). Laying rate was significantly reduced in the group treated with 12,000 mg GAA/kg feed (87% vs 98% in the control group). Average egg weight was significantly reduced in the groups treated with 1,200 and 12,000 mg GAA/kg feed (60.2 and 59.7 g vs 62.4 g in control group) and daily egg mass was significantly reduced in groups treated with 1,200, 6,000 and 12,000 mg GAA/kg feed (57.2, 57.2 and 51.8 vs 61.0 g/day in control group). No differences were reported in yolk or eggshell weights. Regarding egg yolk colour, there were differences between treatments but not dose related. No differences in haematological or clinical chemistry parameters were observed (except albumin, cholesterol and triglycerides that were significantly decreased and glucose that was significantly increased in the 10× group). Creatinine in plasma was below the LOQ in all samples. Plasmatic GAA and creatine increased in a dose-related manner. Analytical values were 10, 30, 59 and 175 µmol GAA/L, for the treatments with 1,200, 3,600, 6,000 and 12,000mg/kg, respectively, vs. 0.4 µmol/L in control group; and 2.2, 3.2, 4.0 and 5.5 mg creatine/dL, respectively, vs. 1.6 mg/dL in control group. Homocysteine in plasma was significantly increased in the groups receiving 3,600, 6,000 and 12,000 mg GAA/kg feed (analytical values 23, 25 and 32 µmol homocysteine/L, respectively, vs. 13 µmol/L in control group). No relevant necropsy or histopathological findings were observed.

The results of the study showed statistically significant negative effects of the GAA supplementation from the maximum proposed use level of 1,200 mg GAA/kg feed on daily feed intake, average egg weight and daily egg mass.

3.2.3.2. Fish

Nile tilapia (*Oreochromis niloticus*) were fed diets containing GAA up to 1.5× the maximum use level for 60 days (Aziza et al., 2020). Growth performance, biochemical parameters and antioxidant capacity were measured. Although the diet contained 20% fishmeal, the supplementation of the diet with 600, 1,200 and 1,800 mg GAA/kg feed resulted in a dose-related improvement of the final body weight, reaching significance at the two highest levels. Specific growth ratio of fish treated at 1,800 mg/kg was not significantly different compared to the control. Body composition was unaffected with the exemption of lipid % that decreased at the highest GAA level. Serum creatine kinase was significantly increased in all GAA-treated groups, creatinine and cholesterol in the 1,200 and 1,800 mg GAA/kg diet groups and triglycerides only in the group receiving 1,800 mg GAA/kg feed in comparison with the control diet. There were no differences between diets regarding the following blood parameters: total protein, globulin, albumin or albumin:globulin. However, the short duration of the study, the low range of experimental doses tested and the inadequacy of the endpoints assessed prevented to reach a conclusion on the safety of GAA for tilapia.

3.2.3.3. Conclusions on safety for the target species

No new data were submitted that would lead the Panel to modify its previous conclusions that GAA at 1,200 mg/kg complete feed is safe for chickens for fattening, piglets and pigs for fattening. This concentration in complete feed would correspond to maximum concentrations in water for drinking of 600 mg GAA/L for chickens for fattening, piglets and pigs for fattening.

The new tolerance study submitted in laying hens indicates that 1,200 mg/kg is not tolerated. Therefore, the Panel is not in a position to conclude on a safe level of GAA in laying/reproductive birds.

In the absence of data on ruminants and salmonids, the FEEDAP Panel cannot conclude on the safety of GAA for all animal species.

3.2.4. Safety for the consumer

3.2.4.1. Residue studies

The previous opinions assessed the deposition in tissues of GAA, its metabolites creatine and creatinine and homocysteine based on data from four studies in chickens for fattening (EFSA, 2009) or on data from nine studies on chickens for fattening and one in pigs (EFSA FEEDAP Panel, 2016). The FEEDAP Panel concluded that there is no concern on consumer safety resulting from the use of GAA in feed for chickens for fattening and piglets/pigs for fattening at 1,200 mg GAA/kg feed.

No new data were submitted that would modify the previous observations on concentrations of GAA, creatine and creatinine in chicken muscle and liver (EFSA FEEDAP Panel, 2016).

As the current application includes the extension of use to all animal species (including ruminants, laying hens and fish), the potential exposure of the consumer through tissues and products from these animals should be estimated. The applicant provided data on deposition in eggs of laying hens and quails, and in fish. However, the studies in fish were not considered adequate as they tested levels far above the maximum proposed use level and did not measure the levels of homocysteine.

No data on deposition of GAA or its metabolites in tissues or milk from ruminants were submitted. The applicant proposed to assess exposure via milk by comparing the data on residues in milk from sows fed diets supplemented with GAA (see Section 3.3.2) with that of milk obtained from animals of other species fed diets not supplemented with GAA.²⁸ The FEEDAP Panel considers that the data from sows cannot be used as surrogate for cow's milk due to the differences in feeding conditions and GAA metabolism between cattle and pigs.

In the tolerance study in laying hens (Section 3.2.3.1),²⁵ GAA, creatine and creatinine levels were analysed at the end of the 56-day experimental period.

Table 1: Deposition of GAA and its metabolites in eggs of hens fed with GAA at 1,200 mg/kg complete feed. Values in mg/kg egg, considering 25% DM in egg

GAA, mg/kg feed	0	1,200
GAA	0.20	0.28
Creatine	4.00 ^a	5.50 ^b
Creatinine	0.43	0.5
Homocysteine	0.90	1.08

Different superscripts in a row are indicative of significant differences ($p < 0.05$).

Supplementing GAA up to the maximum use level did not result in a significant increase in GAA, creatinine or homocysteine deposition in eggs (Table 1). However, creatine deposition was significantly increased with GAA supplementation.

Murakami et al. (2014) analysed the contents of GAA, creatine and creatinine in eggs of quails that had been fed with GAA at 0 and 1,200 mg GAA/kg complete feed for 4 weeks. Supplementation with GAA caused marginal increases in GAA (0 vs. 1.5 $\mu\text{g}/\text{egg}$) and creatine (19.6 vs. 24.1 $\mu\text{g}/\text{egg}$) concentrations in eggs in comparison with the not supplemented control.

3.2.4.2. Assessment of Consumer exposure

In the previous opinion, the Panel concluded that no additional exposure of the consumer to GAA, creatine, creatinine and homocysteine was expected from the use of GAA in feed for chickens for fattening and piglets up to 1,200 mg/kg feed (EFSA FEEDAP Panel, 2016).

A similar conclusion can be reached based on the results observed in deposition of GAA, creatinine and homocysteine in eggs from laying hens fed GAA at 1,200 mg/kg complete feed. Although creatine concentration in eggs was significantly increased with regard to the control, these levels would be in the range (3.3–5.9 mg/kg egg) reported in the scientific literature (Comert and Gokmen, 2020; Reicher et al., 2020).²⁹ The marginal increases in deposition observed in quail eggs are not considered to appreciably increase the exposure of consumers.

In the absence of data in milk and tissues from fish, no conclusion can be reached on the exposure of consumers to GAA or its metabolites from the use of GAA in ruminants and fish.

3.2.4.3. Conclusions on safety for the consumer

The FEEDAP Panel concludes that there is no concern for consumer safety resulting from the use of GAA in feed for chickens for fattening, piglets/pigs for fattening and laying hens under the proposed conditions of use (1,200 mg/kg feed). Due to the limited data available, the FEEDAP Panel cannot conclude on the safety of the additive for the consumer when included in feeds for ruminants and fish.

²⁸ Technical dossier/Supplementary information August 2021/Annex III.3.2.1.2.1.

²⁹ In the study by Reicher et al 2020, the creatine values were expressed in mg/egg freeze dried and they had to be converted to mg/kg egg. Default values used to transform units reported into mg/kg fresh egg: moisture in whole egg is 75%, in egg white is 88% and in egg yolk is 51%; mass of whole edible egg consists of 66% white egg and 34% egg yolk. Molecular weight of creatine = 131.13 g/mol.

3.2.5. Safety for the user

The highest dusting potential measured for GAA (crystalline form) was 7.5 g/m³ and its particle size distribution showed that practically all particles had a diameter < 100 µm. The respective values for the formulated additive were 6.2 g/m³ and 4% of particles < 100 µm diameter. Based on these data, the FEEDAP Panel considered that the exposure of users through inhalation is likely.

In a valid acute inhalation toxicity study performed according to OECD Test Guideline 403 and compliant with good laboratory practice (GLP), the active substance GAA showed an inhalation median lethal dose > 5.1 mg/L air.³⁰

The studies on skin and eye irritancy and on skin sensitisation submitted were already assessed in the former opinion (EFSA, 2009). GAA was considered not irritant to eyes and skin and not a dermal sensitiser. No new data have been submitted that would lead to reconsider the conclusions previously reached.

3.2.5.1. Conclusions on safety for the user

Based on the data available, the FEEDAP Panel concludes that GAA is not toxic by inhalation, it is not an irritant to skin and eyes, and it is not a dermal sensitiser.

3.2.6. Safety for the environment

The safety for the environment was assessed in a previous opinion (EFSA, 2009) and the extension of use to new species is not expected to modify the outcome of the assessment. GAA is a physiological molecule that occurs naturally in animals, and its use as a feed additive would not lead to excretion of molecules which are not normally present in the excreta of the target animals. The FEEDAP Panel concludes that the use of GAA as a feed additive is not expected to pose a risk to the environment.

3.3. Efficacy

The applicant requested the authorisation of GAA as a nutritional additive, functional group amino acids, their salts and analogues. In the view of the FEEDAP Panel, this functional group describes substances which finally enter the metabolism of the body as amino acids and as such take part in the protein synthesis pathways. GAA does not play a comparable role. Although resulting from the amino acid metabolism, GAA is exclusively converted to creatine and cannot be converted back to an amino acid. Therefore, the Panel considers that to support the efficacy of this product as a novel nutritional additive, relevant evidence of its efficacy should be provided at least in poultry, pigs, ruminants and fish, covering both growing and reproductive animals.

The applicant conducted an extensive literature search (described under Section 3.2). From a total of 885 references retrieved, the applicant selected 53 (2 of them EFSA opinions) as relevant after screening of title and abstract and the review of the full text. Eleven additional scientific papers originating from other sources (unspecified) were also included in the list of relevant references.³¹

3.3.1. Poultry

The FEEDAP Panel concluded in a previous opinion (EFSA FEEDAP Panel, 2016) that GAA is able to improve the performance in chickens for fattening at the minimum concentration of 600 mg/kg complete feed. In the absence of adequate data, the FEEDAP Panel could not conclude on the efficacy of GAA in breeder hens and roosters.

From the 48 publications provided by the applicant, nine were considered relevant. The rest were not further considered because there were some deficiencies in the study design or execution (e.g. short duration of the treatment, or the effect was studied in conditions of hypoxia, or there were no replicates, or the control group did not perform as expected, or doses tested were outside the recommended range).

Several studies in chickens for fattening (e.g. Metwally et al., 2015; Malins et al., 2017; Yapontsev et al., 2017; and Córdova-Noboa et al., 2018) support the previous conclusion on the lowest effective dose of GAA (600 mg/kg complete feed) (EFSA FEEDAP Panel, 2016).

³⁰ Technical dossier/Section III/Annex III.3.2.2.1.

³¹ Technical dossier/Section IV.4.3.

Ringel et al. (2007) studied the effect of supplementing GAA at 400 or 800 mg GAA/kg feed for 20 weeks on the performance of turkeys for fattening. The supplementation with a concentration of 800 mg GAA/kg feed significantly reduced feed intake and improved feed to gain ratio (2.66 vs. 2.72), with no improvements in the body weight gain. Carcass weight and yield were unaffected by treatments. Breast weight percentage was higher in turkeys fed 400 or 800 mg GAA/kg and leg weight percentage was lower at 800 mg GAA/kg.

Morshedy et al. (2019) studied the effect of supplementing GAA at 500 mg/kg feed with or without supplemental methionine on the performance of ducks for fattening and meat parameters. GAA supplemented at 500 mg/kg feed for 42 days improved ducks' performance parameters (body weight (BW), body weight gain (BWG), feed to gain ratio and protein efficiency ratio) and carcass traits.

Kuhi Darmani et al. (2019) studied the effect of supplementing GAA at 600 or 1,200 mg/kg feed and L-arginine for 42 days on performance, blood metabolites and carcass characteristics of Japanese quails. Supplementing a diet covering arginine requirements for quails for fattening with 600 or 1,200 mg GAA/kg feed had no effect on performance parameters (feed intake (FI), BWG and feed to gain ratio) or carcass traits.

Khakran et al. (2017) studied the effect of supplementing GAA ranging from 570 to 1,710 mg/kg feed for 84 days on productive performance, some blood biochemical parameters and reproductive hormones of laying hens. No significant effects were seen in the feed intake, laying rate, egg mass and feed to egg mass ratio. Supplementation at 1,710 mg GAA/kg feed caused a reduction in egg weight.

Tapeh et al. (2017) studied the effect of supplementing GAA diet supplementation on semen quality and fertility of breeder roosters for chickens for fattening when added from 600 to 1,800 mg/kg feed for 26 weeks. Supplementing complete feed with 1,200 mg GAA/kg showed improvements in the reproductive capacity of roosters. However, as concurrent negative and positive effects were reported at the 600 mg/kg (lower sperm concentration, higher fertility), a clear conclusion at the recommended levels cannot be drawn.

3.3.1.1. Conclusions on efficacy in poultry

Based on the new studies provided, the Panel confirms its previous conclusion that 600 mg GAA/kg feed is efficacious in improving the performance of chickens for fattening. Similar results were observed in turkeys for fattening (800 mg/kg) and ducks (500 mg/kg), but not in quail. Considering the overall data, the Panel concludes that GAA at a minimum level of 600 mg/kg complete feed has the potential to improve the zootechnical performance of all growing avian species. No conclusion can be reached on reproductive avian species.

3.3.2. Pigs

As regards growing pigs, one valid study on the efficacy of GAA in weaned piglets submitted in a previous opinion showed that 1,200 mg/kg complete feed improved growth (final BW and average daily gain (ADG)) (EFSA FEEDAP Panel, 2016). The applicant submitted eight new studies. Six of them were not considered further due to the short duration of the trials.

He et al. (2018) studied the effects of dietary GAA supplementation at 300, 900 and 1,200 mg/kg feed for 14 weeks on growth performance, creatine and energy metabolism, and carcass characteristics in grower–finisher pigs. GAA supplementation significantly improved gain to feed ratio at 300, 900 and 1,200 mg GAA/kg feed; and the hot carcass weight at 300 and 1,200 mg/kg. GAA treatments did not significantly affect serum (GAA, creatine, creatinine and creatine kinase [CK]) or tissue concentrations (GAA, creatine in muscle, liver or kidney, and CK in muscle) of energy-related metabolites, except serum ATP which was significantly increased in a dose-dependent manner with the GAA feed content.

Jayaraman et al. (2018) studied the effect of the dietary supplementation of 800 or 1,200 mg GAA/kg feed to pig diets for 150 days on performance, carcass characteristics and meat quality. Performance parameters (final BW, ADG and feed to gain ratio) were significantly improved with GAA supplementation at any level. Some carcass parameters (% of lean meat, back fat) were significantly improved at GAA supplementation of 1,200 mg/kg.

Considering pigs for reproduction, the applicant provided a new study in pregnant sows and their progeny of 42-day duration (from gestation day 100 to weaning – day 28 post-farrowing).²⁸ The basal diets (gestation and lactation) were either not supplemented (control) or supplemented with 1,000, 5,000 or 10,000 mg GAA/kg feed (confirmed by analysis). No relevant significant differences between

treatments were observed in sows' performance parameters (percentage of BW loss in lactation, percentage of back fat loss in lactation, feed consumption (FI)); or in piglets performance parameters (final BW, litter homogeneity, ADG, pre-weaning mortality).

Mendonça et al. (2019) studied the effect of the dietary supplementation with 1,000 mg GAA/kg feed for sows and their progenies (one production cycle) in nursery pigs' performance and blood parameters. Data on reproductive performance of sows (e.g. reproductive data, total born, weaned pigs/sow) were not provided. Dietary supplementation with 1,000 mg GAA/kg of sows and their progenies did not affect performance parameters (average daily feed intake, ADG and feed to gain) and creatinine and creatine kinase serum concentrations of piglets at nursery.

3.3.2.1. Conclusions on the efficacy in pigs

Considering the three studies in which positive effects on performance of growing pigs were observed at levels ranging 300–1,200 mg GAA/kg feed, the Panel concludes that the dietary supplementation of GAA under the proposed conditions of use has the potential to improve the zootechnical performance in growing *Suidae*. In the absence of adequate evidence, no conclusions can be reached regarding the efficacy in reproductive pigs.

3.3.3. Ruminants

As regards growing ruminants, Li et al. (2020) studied the effect of the dietary supplementation with GAA from 300 to 900 mg/kg DM on the performance, nutrient digestion, rumen fermentation and blood metabolites in Angus bulls of 1 year of age. Significant improvements in ADG and final BW were seen in all treated groups and DM intake and feed to gain significantly improved at 600 and 900 mg/kg DM.

Chao et al. (2019) studied the effect of the dietary supplementation with GAA from 400 to 1,200 mg/kg feed for 85 days on growth performance, slaughter performance, fat deposition and nutritional components in muscle of Chinese Tan rams. The supplementation of weaned Tan rams' diet with 800 mg GAA/kg feed improved performance (final BW, ADG, feed to gain ratio), carcass characteristics (carcass yield, carcass' lean meat percentage, tail fat, subcutaneous fat thickness and marbling score) and nutritional composition of meat (glycogen, protein and intramuscular fat content) in comparison with the control diet.

3.3.3.1. Conclusions on the efficacy in ruminants

The dietary supplementation with a range of 300–900 mg GAA/kg DM feed is considered efficacious for improving the zootechnical performance of growing ruminants. In the absence of data, no conclusions can be reached for young (milk/milk replacer fed) ruminants and for reproductive ruminants.

3.3.4. Aquatic animal species

The applicant submitted scientific papers in relation to the efficacy of the additive in Nile tilapia (*Oreochromis niloticus*), Jian carps (*Cyprinus carpio* var. Jian) and North American bullfrog (*Lithobates catesbeianus*).

Aziza et al. (2019) assessed the effect of supplementing 600, 1,200 and 1,800 mg GAA/kg feed for 60 days on Nile tilapia's (*Oreochromis niloticus*) growth performance. Body weight gain improved with supplementation levels above 600 mg GAA/kg feed. Final body weight, body weight gain and protein efficiency ratio improved at 1,200 and 1,800 mg GAA/kg feed. Specific growth rate was increased at 1,200 mg GAA/kg feed. Dietary supplementation of Nile tilapia's feed with 1,200 and 1,800 mg GAA/kg feed improved performance parameters.

Fu et al. (2015) studied the effect of supplementing 250, 500 and 1,000 mg GAA/kg feed for 42 days on growth performance, and body composition in Jian carps (*Cyprinus carpio* var. Jian). The supplementation of 250 and 500 mg of GAA/kg feed improved feed to gain ratio. No other differences were seen in performance parameters or carcass composition, except for an increased viscera relative weight in the groups treated with GAA.

Zeng et al. (2018) studied the effect of supplementing 200, 400, 600 and 800 mg GAA/kg feed for 53 days in all-plant protein diets on growth and muscle energy metabolism of North American bullfrog (*Lithobates catesbeianus*). The supplementation of 200 mg GAA/kg feed improved protein efficiency ratio in comparison with the control diet. The supplementation of 400 mg of GAA/kg feed improved final BW, weight gain, specific growth rate, feed to gain ratio and protein efficiency ratio in comparison

with the control diet. As regards carcass indexes, in comparison with the control, all treatments decreased the hepatosomatic index, and 600 and 800 mg GAA/kg increased hind leg index.

3.3.4.1. Conclusions on the efficacy in aquatic animal species

The dietary supplementation with GAA at the proposed use range (600–1,200 mg GAA/kg) has the potential to improve zootechnical performance of growing fish other than salmonids. Even lower GAA levels seem to be efficacious in carp and bullfrog. The FEEDAP Panel cannot conclude on the efficacy of the additive under assessment for salmonids or for crustaceans.

3.3.5. Conclusions on efficacy

The use of the additive under assessment in animal nutrition at the proposed conditions of use has the potential to be efficacious in all growing avian, *Suidae*, ruminant (except for pre-ruminants) species; in growing fin fish other than salmonids and in frog. It is not possible to conclude on the efficacy of the additive in other species, and in reproductive animals.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation³² and Good Manufacturing Practice.

4. Conclusions

The FEEDAP Panel concludes that GAA at 1,200 mg/kg complete feed is safe for chickens for fattening, piglets and pigs for fattening. This concentration in complete feed would correspond to maximum concentrations in water of 600 mg GAA/L for chickens for fattening, piglets and pigs for fattening. The Panel is not in a position to conclude on a safe level of GAA in laying/reproductive birds. In the absence of data on ruminants and salmonids, the FEEDAP Panel cannot conclude on the safety of GAA for all animal species.

There is no concern on consumer safety resulting from the use of GAA in feed for poultry and pigs at the proposed conditions of use. The limited data do not allow to conclude on the safety for the consumer when the additive is used in feed for ruminants or fish.

GAA is not toxic by inhalation, it is not an irritant to skin and eyes and it is not a dermal sensitiser.

The FEEDAP Panel concludes that the use of GAA as feed additive is not expected to pose a risk to the environment.

The use of the additive under assessment in animal nutrition at the proposed conditions of use has the potential to be efficacious in all growing avian, *Suidae*, ruminant (except for pre-ruminants) species; in growing fin fish other than salmonids and in frog. It is not possible to conclude on the efficacy of the additive in other species, and in reproductive animals.

5. Recommendation

The maximum safe levels of GAA in feed for chickens for fattening, piglets and pigs for fattening are derived under the assumption that the feed contains sufficient amounts of methyl donors (other than methionine, e.g. choline, betaine and folic acid) and vitamin B₁₂. It is recommended to include the corresponding statement in the 'other provisions' of the authorisation.

6. Documentation provided to EFSA/Chronology

Date	Event
30/06/2020	Dossier received by EFSA. Guanidinoacetic acid for all animal species. Alzchem Trostberg GmbH.
17/07/2020	Reception mandate from the European Commission
28/09/2020	Application validated by EFSA – Start of the scientific assessment

³² Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 268, 8.2.2005, p. 1.

Date	Event
30/11/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: Characterisation of the additive, conditions of use, safety for the consumer.</i>
05/01/2021	Comments received from Member States
24/08/2021	Reception of supplementary information from the applicant - Scientific assessment re-started
24/03/2022	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
ADI	acceptable daily intake
AFC	EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
ANS	EFSA Scientific Panel on Additives and Nutrient Sources added to Food
BW	body weight
CAS	Chemical Abstracts Service
CFU	colony-forming unit
CV	coefficient of variation
DM	dry matter
EINECS	European Inventory of Existing Chemical Substances
EURL	European Union Reference Laboratory
FCR	feed conversion ratio
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
IUPAC	International Union of Pure and Applied Chemistry
LOD	limit of detection
LOQ	limit of quantification
MRL	maximum residue limit
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development