Supplementation of mixed doses of glutamate and glutamine can improve the growth and gut health of piglets during the first 2 weeks post-weaning

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The aim of this study was to test the effect of mixing doses of glutamate (Glu) and glutamine (Gln) on the growth, health and gut health of post-weaning piglets. One hundred twenty weaned piglets (24 ± 2 days of age) were assigned to 6 dietary groups: (1) standard diet (CO); (2) CO plus Glu (6 kg/Ton): 100Glu; (3) CO plus 75Glu + 25Gln; (4) CO plus 50Glu + 50Gln; (5) CO plus 25Glu + 75Gln and (6) CO plus 100Gln. At days 8 and 21, blood was collected for haematological and reactive oxygen metabolite analysis, intestinal mucosa for morphological and gene expression analysis, and caecal content for microbial analysis. Data were fitted using a Generalised Linear Model (GLM). Piglet growth increased linearly with an increase in Gln from d7 to d14. The Glu:Gln ratio had a quadratic effect on faecal consistency and days of diarrhoea, neutrophil% and lymphocyte%, and a positive linear effect on monocyte% in the blood at d8. The amino acids (AAs) reduced the intraepithelial lymphocytes in the jejunum, and 100Gln improved intestinal barrier integrity at d8. The caecal microbiota did not differ. Overall, this study suggested a favourable effect of mixing Glu and Gln (25 + 75–50 + 50) as a dietary supplementation in post-weaning piglets to benefit the immune and barrier function of the gut, resulting in an increase in faecal consistency and improvement of growth during the first 2 weeks post-weaning.

Amino acids (AAs) are not only needed for protein production but can be considered precursors of energy, signalling molecules and microbiota modulators. Therefore, AAs can contribute to restoring gut health and, in turn, improving general health. L-Glutamate (Glu) and L-Glutamine (Gln) are abundant AAs in the body and have traditionally been considered dispensable AAs. However, they are currently regarded as a conditionally indispensable nutrient under stress conditions, including the weaning of piglets. These two AAs can benefit the gut health of piglets by acting as sources of energy for the intestinal cells, as precursors for promoting cell proliferation in the gut and as precursors for the immune cells. Glutamate is mainly utilised as a source of energy by enterocytes. However, it can be used as a precursor for the synthesis of Gln and glutathione; thus, it plays a key role in preserving the gut from oxidative damage.

The beneficial effects of Gln on the gut are mainly related to its use as a source of energy for enterocytes3, as metabolic fuel for the immune cells (including lymphocytes and macrophages) and by supplying substrates for the synthesis of glucosamines, nucleic acids, nucleotides and adenosine triphosphate (ATP)4–6. Furthermore, Gln can modulate the phosphorylation of the mammalian target of rapamycin (mTOR) which is involved in the regulation of protein synthesis in the intestine6.

Previous studies have shown that the supplementation of Glu alone and Gln alone could improve the growth performance, and gut health of weaning piglets in terms of gut integrity, acting as modulators of the mucosal gene expression and gut microbial community10–14.

*The metabolism of Glu and Gln is closely connected; in fact, Glu is the immediate product of the Gln metabolism, produced by the action of glutaminase; Gln can be combined with ammonia (NH3) to produce Glu by the
action of glutamine synthetase in some tissues and cells, such as the liver and skeletal muscles. It is plausible that the combined supplementation of Glu and Gln could have synergistic effects. However, to date, only a few studies have investigated the effect of mixing Glu and Gln supplementation on piglets, suggesting a positive effect on growth performance and gut morphology. Up to now, no study has investigated the effect of mixing Glu and Gln at different doses regarding the growth, health and gut eubiosis of post-weaning piglets. Therefore, in the present study, the hypothesis that mixing different doses of Glu and Gln affected the growth, immune response and gut health of post-weaning piglets was tested. The aims of the present study were to (1) investigate the various beneficial effects of mixing different doses of Glu and Gln on the intestinal health and growth of post-weaning piglets and to evaluate whether mixing doses of Glu and Gln could be more promising than providing a single AA, (2) elucidate the mode of action of mixing different doses of Glu and Gln on the gut health of post-weaning piglets and (3) identify the best supplementation ratio of Glu and Gln for sustaining piglets during the post-weaning phase.

Results
To test the hypothesis that mixing different doses of Glu and Gln affected the growth, immune response and gut health of post-weaning piglets an in vivo trial was carried out; the piglets were assigned to 6 groups fed (1) a standard diet (CO), (2) CO plus 6 kg/Ton of Glu alone (100Glu), (3) CO plus 75% of Glu and 25% of Gln (75Glu + 25Gln), (4) CO plus 50% of Glu and Gln (50Glu + 50Gln), (5) CO plus 25% of Glu and 75% of Gln (25Glu + 75Gln) and (6) CO plus 6 kg/Ton Gln (100Gln). Significant values are in bold.

Table 1. Effect of the dietary supplementation (6 kg/T) with glutamate and glutamine in different ratio on growth performance of post-weaning piglets. Diet 1 CO = standard diet; 100Glu = CO plus 6 kg/Ton Glu; 75Glu + 25Gln = CO plus 4.5 kg/Ton Glu and 1.5 kg/Ton Gln; 50Glu + 50Gln = CO plus 3 kg/Ton Glu and 3 kg/Ton Gln; 25Glu + 75Gln = CO plus 1.5 kg/Ton Glu and 4.5 kg/Ton Gln; 100Gln = CO plus 6 kg/Ton Gln. Significant values are in bold.

| Item                   | CO       | 100Glu   | 75Glu + 25Gln | 50Glu + 50Gln | 25Glu + 75Gln | 100Gln |
|------------------------|----------|----------|---------------|---------------|---------------|--------|
|                       | SEM      | Diet     | Linear        | Quadratic     | CO vs AAs addition | 100Glu vs mixed addition | 100Gln vs mixed addition |
| Body weight, kg        |          |          |               |               |                |        |        |
| d0                     | 6.94     | 6.98     | 6.77          | 7.00          | 7.07           | 0.21   | 0.932  | 0.393  | 0.935  | 0.807  | 0.756  | 0.664  |
| d7                     | 7.79     | 7.63     | 7.53          | 7.86          | 7.77           | 0.24   | 0.914  | 0.486  | 0.533  | 0.434  | 0.350  | 0.769  |
| d8                     | 7.57     | 7.57     | 7.61          | 7.90          | 7.48           | 0.46   | 0.821  | 0.468  | 0.804  | 0.689  | 0.820  | 0.352  |
| d14                    | 8.48     | 8.45     | 8.27          | 8.96          | 9.52           | 0.47   | 0.441  | 0.152  | 0.566  | 0.473  | 0.383  | 0.995  |
| d21                    | 11.01    | 10.33    | 10.47         | 10.82         | 11.76          | 0.52   | 0.413  | 0.065  | 0.720  | 0.875  | 0.255  | 0.701  |
| Average daily gain, kg/day |          |          |               |               |                |        |        |
| d0–d7                  | 0.12     | 0.09     | 0.11          | 0.10          | 0.12           | 0.02   | 0.704  | 0.486  | 0.534  | 0.434  | 0.350  | 0.769  |
| d7–d14                 | 0.10     | 0.14     | 0.15          | 0.20          | 0.21           | 0.04   | 0.234  | 0.201  | 0.444  | 0.050  | 0.300  | 0.980  |
| d0–d14                 | 0.11     | 0.12     | 0.16          | 0.16          | 0.18           | 0.02   | 0.265  | 0.126  | 0.323  | 0.115  | 0.167  | 0.917  |
| d14–d21                | 0.36     | 0.27     | 0.31          | 0.37          | 0.32           | 0.02   | 0.026  | 0.073  | 0.620  | 0.015  | 0.257  | 0.221  |
| d0–d21                 | 0.19     | 0.17     | 0.19          | 0.20          | 0.22           | 0.02   | 0.427  | 0.051  | 0.563  | 0.839  | 0.118  | 0.663  |
| Daily feed intake, kg/day |          |          |               |               |                |        |        |
| d0–d7                  | 0.15     | 0.15     | 0.16          | 0.16          | 0.15           | 0.01   | 0.962  | 0.600  | 0.495  | 0.689  | 0.369  | 0.890  |
| d7–d14                 | 0.30     | 0.32     | 0.30          | 0.35          | 0.36           | 0.02   | 0.271  | 0.239  | 0.447  | 0.142  | 0.436  | 0.832  |
| d14–d21                | 0.51     | 0.44     | 0.48          | 0.48          | 0.51           | 0.03   | 0.459  | 0.069  | 0.881  | 0.399  | 0.188  | 0.397  |
| d7–d21                 | 0.41     | 0.38     | 0.39          | 0.41          | 0.44           | 0.03   | 0.610  | 0.089  | 0.656  | 0.889  | 0.230  | 0.654  |
| Gain to feed ratio     |          |          |               |               |                |        |        |
| d0–d7                  | 0.08     | 0.10     | 0.10          | 0.11          | 0.09           | 0.01   | 0.572  | 0.527  | 0.286  | 0.434  | 0.471  | 0.371  |
| d7–d14                 | 0.07     | 0.12     | 0.04          | 0.31          | 0.19           | 0.01   | 0.613  | 0.821  | 0.526  | 0.486  | 0.813  | 0.853  | 0.246  |
| d14–d21                | 0.95     | 0.84     | 0.85          | 0.70          | 1.05           | 0.63   | 0.818  | 0.772  | 0.653  | 0.556  | 0.902  | 0.384  |
| d7–d21                 | 0.56     | 0.52     | 0.56          | 0.56          | 0.60           | 0.01   | 0.300  | 0.051  | 0.711  | 0.570  | 0.078  | 0.447  |
From d0 to d21 a linear effect of the Glu-Gln ratio was observed for the ADG with a favourable effect of the highest dose of Gln \((P = 0.051)\). The feed intake (FI) was not affected by diet for the periods d0–d7 or d7–d14. In the periods d14–d21 and d7–d21, the FI tended to have a linear effect on the Glu:Gln ratio, which increased the value of the highest dose of Gln \((P = 0.069\) and \(P = 0.089\), respectively). The gain to feed (G:F) ratio was not affected by diet for the periods d0–d7, d7–d14 or d14–d21. From d7 to d21, the G:F ratio tended to have a linear effect on the Glu:Gln ratio \((P = 0.051)\), and the 100 Glu group tended to have a lower G:F ratio than the other groups supplemented with mixed doses of AAs \((P = 0.078)\).

Blood parameters. Table 2 shows the effect of the dietary supplementation on blood values at d8. No difference was observed for the values of haemoglobin (HGB), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelets and the percentage of red cell distribution width (RDW) in the number of leukocytes, neutrophils, lymphocytes, basophils and the percentage of basophils. The level of the red blood cell (RBC) count tended to be higher in the Glu alone group (100Glu) as compared with the other groups supplemented with mixed doses of AAs \((P = 0.059)\). For hematocrit % (HCT %), a quadratic effect
Table 2. Effect of the dietary supplementation (6 kg/T) with glutamate and glutamine in different ratio on haematological parameters of piglets at 8 days post-weaning. 1 RBC, red blood cells, HGB, haemoglobin, HCT, haematocrit, MCV, mean corpuscular volume, MCH, mean corpuscular haemoglobin, MCHC, mean corpuscular haemoglobin concentration, RDW, red cell distribution width. Diet 2 CO = standard diet; 100Glu = CO plus 6 kg/Ton Glu; 75Glu + 25Gln = CO plus 4.5 kg/Ton Glu and 1.5 kg/Ton Gln; 50Glu + 50Gln = CO plus 3 kg/Ton Glu and 3 kg/Ton Gln; 25Glu + 75Gln = CO plus 1.5 kg/Ton Glu and 4.5 kg/Ton Gln; 100Gln = CO plus 6 kg/Ton Gln. Significant values are in bold.

| Item 3 | CO  | 100Glu | 75Glu + 25Gln | 50Glu + 50Gln | 25Glu + 75Gln | 100Gln | SEM | Diet 4 | Linear | Quadratic | CO vs AAs addition | 100Glu vs mixed addition | 100Gln vs mixed addition |
|--------|-----|--------|---------------|---------------|---------------|--------|-----|--------|--------|-----------|----------------------|------------------------|------------------------|
| RBC, M/µL | 6.92 | 7.07 | 6.81 | 6.75 | 6.76 | 6.8 | 0.1 | 0.530 | 0.180 | 0.181 | 0.552 | 0.059 | 0.869 |
| HGB g/dL | 11.9 | 12 | 11.6 | 11.7 | 11.7 | 11.8 | 0.2 | 0.708 | 0.567 | 0.142 | 0.687 | 0.108 | 0.458 |
| HCT, % | 37.8 | 38.1 | 36.3 | 36.6 | 36.5 | 37.6 | 0.6 | 0.201 | 0.680 | 0.023 | 0.291 | 0.025 | 0.130 |
| MCV, fl | 54.8 | 54.3 | 53.3 | 54.4 | 54.1 | 55.4 | 0.6 | 0.326 | 0.156 | 0.172 | 0.499 | 0.604 | 0.049 |
| MCHC, g/dL | 15.66 | 15.07 | 13.76 | 13.92 | 13.76 | 14.07 | 0.9 | 0.457 | 0.976 | 0.797 | 0.234 | 0.717 | 0.555 |
| MCH, pg | 17.2 | 17.2 | 17.1 | 17.1 | 17.3 | 17.5 | 0.3 | 0.946 | 0.332 | 0.819 | 0.884 | 0.771 | 0.438 |
| RDW , % | 25.4 | 25.4 | 24.5 | 24.9 | 25.1 | 24.6 | 0.8 | 0.947 | 0.696 | 0.866 | 0.583 | 0.766 | 0.553 |
| Leukocytes, K/µL | 600 | 614 | 665 | 668 | 640 | 580 | 44 | 0.638 | 0.515 | 0.121 | 0.473 | 0.386 | 0.130 |
| Eosinophils, K/µL | 0.46 | 0.59 | 0.44 | 0.45 | 0.54 | 0.32 | 0.08 | 0.178 | 0.068 | 0.783 | 0.943 | 0.196 | 0.063 |
| Neutrophils, K/µL | 7.46 | 7.6 | 6.4 | 5.47 | 7.1 | 6.85 | 0.57 | 0.136 | 0.912 | 0.069 | 0.752 | 0.139 | 0.427 |
| Lymphocytes, K/µL | 20.3 | 12.1 | 20.4 | 13.9 | 21.1 | 21.2 | 0.23 | 0.010 | 0.012 | 0.764 | 0.297 | 0.279 | 0.012 |
| Monocytes, K/µL | 0.31 | 0.19 | 0.27 | 0.18 | 0.33 | 0.29 | 0.04 | 0.011 | 0.026 | 0.961 | 0.113 | 0.107 | 0.460 |
| Basophils, K/µL | 0.04 | 0.04 | 0.03 | 0.05 | 0.04 | 0.04 | 0.01 | 0.965 | 0.796 | 0.807 | 0.956 | 0.943 | 0.943 |
| Neutrophils, % | 47.35 | 46.16 | 47.71 | 54.8 | 47.5 | 45.87 | 2.36 | 0.075 | 0.918 | 0.012 | 0.675 | 0.159 | 0.131 |
| Lymphocytes, % | 47.14 | 48.12 | 46.44 | 39.66 | 48.85 | 49.29 | 2.53 | 0.104 | 0.738 | 0.012 | 0.695 | 0.195 | 0.092 |
| Monocytes, % | 3.16 | 4.22 | 3.56 | 3.71 | 3.3 | 2.39 | 0.55 | 0.291 | 0.028 | 0.599 | 0.643 | 0.268 | 0.075 |
| Eosinophils, % | 0.32 | 0.29 | 0.27 | 0.4 | 0.24 | 0.33 | 0.07 | 0.718 | 0.815 | 0.839 | 0.822 | 0.910 | 0.724 |
| Basophils, % | 24.07 | 21.75 | 21.07 | 22.63 | 24.12 | 22.57 | 1.29 | 0.487 | 0.260 | 0.703 | 0.236 | 0.564 | 0.980 |
| GPX-1, ng/mL | 557 | 640 | 609 | 545 | 558 | 522 | 61.42 | 0.727 | 0.321 | 0.434 | 0.775 | 0.301 | 0.478 |
| GSH, uM | 0.31 | 0.14 | 0.39 | 0.18 | 0.21 | 0.17 | 0.11 | 0.640 | 0.439 | 0.810 | 0.449 | 0.377 | 0.495 |

of the Glu:Gln ratio (P = 0.023) was observed, and the 100Glu group had a higher value as compared with the other groups supplemented with mixed doses of AAs (P = 0.025). The level of mean corpuscular volume (MCV) was higher in the Gln alone group (100Gln) as compared with the other groups supplemented with mixed doses of AAs (P = 0.049). Considering the white cell fraction, the diet influenced the number and the percentage of eosinophils (P = 0.011; P = 0.010, respectively). For the eosinophil count, a linear effect of the Glu:Gln ratio was observed (P = 0.026). For the percentage of eosinophils, a higher value was observed in the Gln alone group (100Gln) as compared with the other groups supplemented with mixed doses of AAs (P = 0.012), and a negative linear effect of Glu addition was observed (P = 0.012). A trend and a linear effect for the Glu:Gln ratio was observed regarding the number and the percentage of monocytes (P = 0.068; P = 0.028, respectively), and the Gln alone group tended to have a lower value of this white cell fraction as compared with the groups receiving the mixed doses of AAs (P = 0.063; P = 0.075, respectively). The diet tended to affect the percentage of neutrophils (P = 0.075); a quadratic effect of the Glu:Gln ratio was observed (P = 0.012) in which the 50Glu + 50Gln group had the highest value. The diet tended to affect the percentage of lymphocytes (P = 0.01); a quadratic effect of the Glu:Gln ratio was observed (P = 0.012) in which the 50Glu + 50Gln group had the lowest value. In addition, the Gln alone group (100Gln) tended to have a higher lymphocyte as percentage compared with the other groups supplemented with AAs (P = 0.092) (Table 2).

No difference due to diet was observed for any of the parameters, except for HGB for which the CO group tended to have a lower value as compared with the groups supplemented with AAs (P = 0.079) and for the percentage of eosinophils for which the diet showed a trend (P = 0.057); no significant contrasts were determined (Supplementary Table 1).

Caecal microbiota. A total of 4,098,584 quality checked reads on 103 samples were obtained resulting in 3523 different amplicon sequence variants (ASVs). The rarefaction curves relative to all the samples are shown...
in Supplementary Fig. 1. Considering the overall samples, eighteen different phyla were identified in the caecum; the most abundant phylum was Firmicutes (70%) followed by Bacteroidetes (17%). At the family level, eighty different genera were assigned; the most abundant were Peptostreptococcaceae (18%) and Erysipelotrichaceae (17%). At the genus level, the most abundant assigned genera were Turicibacter (15%) and Terrisporobacter (13%). Figure 2 shows the Alpha diversity indices of the dietary groups at the two time points. The dietary groups showed a fairly constant ASVs distribution in the caecum; the Alpha index values (Chao1, Shannon and InvSimpson) were not affected by diet. Time did not influence the Shannon and InvSimpson indices, but reduced the Chao1 index from d8 (265) to d21 (231) \(\left( P = 0.008 \right)\). For the Beta diversity index (Bray–Curtis distance), a clear effect of time was observed \(\left( P = 0.01; \ R^2 = 0.068 \right)\). Two well-defined clusters regarding time were shown in the Non-Metric Multidimensional Scaling (NMDS) plot of the Bray–Curtis distance matrix (Supplementary Fig. 2). Diet did not affect the Beta diversity index. Results for the different taxa of the dietary groups at the family and genera levels at d8 and d21 are reported in Table 3. At d8, the 100Gln group had a lower abundance of family Fusobacteriaceae as compared with the CO group \(\left( adj.p < 0.0001 \right)\); considering the comparison between the Glu alone group vs. the mixed addition groups, the 100Glu group had a lower relative abundance of Enterococcaceae \(\left( adj.p < 0.0001 \right)\), and a higher abundance of Erysipelotrichaceae \(\left( adj.p = 0.02 \right)\). Considering the comparison between the Gln alone group vs. the mixed addition groups, the 100Gln group had a lower abundance of Fusobacteriaceae \(\left( adj.p < 0.0001 \right)\) and a higher level of Clostridiales_vadinBB60_group \(\left( adj.p = 0.001 \right)\). Considering the comparisons at the genus level at d8, the CO group had a higher relative abundance of Selenomonas \(\left( adj.p = 0.007 \right)\) and Mogibacterium \(\left( adj.p < 0.0001 \right)\) than the 100Glu group, a higher relative abundance of Pelistega \(\left( adj.p < 0.0001 \right)\) and Selenomonas \(\left( adj.p = 0.006 \right)\) than the 50Glu + 50Gln group, a higher relative abundance of Selenomonas \(\left( adj.p = 0.004 \right)\) and Blautia \(\left( adj.p = 0.023 \right)\) than the 25Glu + 75Gln group and a higher relative abundance of Fusobacterium \(\left( adj.p < 0.0001 \right)\) and UB1819 (Family Ruminococcaceae) \(\left( adj.p < 0.0001 \right)\) as compared with the 100Gln group. The comparison between the Gln alone group or the Glu alone group vs. the AAs mixed diets showed a decrease in the relative abundance of Pediococcus \(\left( adj.p < 0.0001 \right)\), Enterococcus \(\left( adj.p < 0.0001 \right)\) and Lactobacillus \(\left( adj.p = 0.008 \right)\), in the 100Glu group, and a decrease in Pediococcus \(\left( adj.p < 0.05 \right)\) and Fusobacterium \(\left( adj.p < 0.0001 \right)\) in the 100Gln group. At d21, the CO group had a lower abundance of Fibrobacteraceae vs. each of the groups supplemented with AAs \(\left( adj.p < 0.0001 \right)\) and a lower relative abundance of Bacteroidales_RF16_group \(\left( adj.p = 0.028 \right)\) and Peptococcaceae as compared with the 100Gln group \(\left( adj.p = 0.037 \right)\). Considering the comparisons at the genus level at d21, the CO group had a lower relative abundance of Selenomonas_3 as compared with the 100Glu group \(\left( adj.p = 0.006 \right)\) and the

![Figure 2](https://www.nature.com/scientificreports/)
Intestinal morphology. Table 4 shows the effect of dietary supplementation on the intestinal morphology measurements at d8 and d21. At d8, no difference due to diet was observed for villus height, villus width, crypt depth, and the number of goblet cells and duodenal glands. However, at d8, in the duodenum, the number of duodenal glands was higher in the 100Gln group as compared with the other groups supplemented with mixed doses of AAs ($P = 0.048$); a quadratic effect of the Glu:Gln ratio was observed ($P = 0.048$). At d8, in the jejunum, the number of goblet cells was lower in the CO group as compared with the 50Glu + 50Gln group ($P = 0.05$); in the ileum, the villus height tended to be higher in the 100Glu group as compared with the other groups supplemented with mixed doses of AAs ($P = 0.097$). The villus width tended to be higher in the CO group as compared with the 100Glu group ($P = 0.097$) and the number of goblet cells tended to increase linearly with the Gln dose ($P = 0.090$). No difference due to diet was observed for the interstitial score in any of the intestinal segments at d8. Diet influenced the intraepithelial score in the jejunum at d8; the CO group had a higher probability of having a higher intraepithelial lymphocyte score as compared with the groups supplemented with Glu, Gln or both ($P < 0.016$), and the 25Glu + 75Gln group showed a lower probability of a high intraepithelial lymphocyte score

25Glu + 75Gln groups (adj.$p = 0.007$), and a lower relative abundance of *Succiniclasticum* as compared with the groups supplemented with AAs (adj.$p < 0.0001$).

### Table 3. Effect of the dietary supplementation (6 kg/T) with glutamate and glutamine in different ratio on microbial taxa composition of piglets at 8- and 21-days post-weaning. baseMean$^1$ = mean of normalized taxa counts averaged over all samples from both conditions. log2FoldChange$^2$ = log2 Fold Change. The sign is relative to the first group identified in the comparison. lfcSE$^3$ = log2 Fold change standard error. P value$^4$ = Wald statistic value. Padj$^5$ = Benjamini–Hochberg adjusted p value.

| Comparison                                      | Base Mean$^1$ | log2 Fold Change$^2$ | lfcSE$^3$ | P value$^4$ | Padj$^5$ | Taxa                                                                 |
|------------------------------------------------|---------------|----------------------|-----------|-------------|-----------|----------------------------------------------------------------------|
| **Day 8**                                       |               |                      |           |             |           |                                                                      |
| **Family level**                                 |               |                      |           |             |           |                                                                      |
| 100Gln vs CO                                    | 22.87         | −21.51               | 2.96      | <0.0001     | <0.0001   | Fusobacteriaceae                                                     |
| Mixed additions vs Glu                          | 10.78         | 21.06                | 3.41      | <0.0001     | <0.0001   | Enterococcaceae                                                      |
|                                                 | 1989.71       | 2.75                 | 0.63      | <0.0001     | <0.0001   | Lactobacillaceae                                                     |
|                                                 | 7931.63       | −2.77                | 0.84      | 0.001       | 0.02      | Erysipelotrichaceae                                                  |
| Mixed additions vs Gln                          | 25.76         | 23.38                | 2.14      | <0.0001     | <0.0001   | Fusobacteriaceae                                                     |
|                                                 | 495.19        | −3.31                | 0.94      | <0.0001     | 0.001     | Clostridiales_vadinBB60_group                                         |
| **Genus level**                                 |               |                      |           |             |           |                                                                      |
| 100Glu vs CO                                    | 19.62         | −21.65               | 2.78      | <0.0001     | <0.0001   | Mogibacterium                                                        |
|                                                 | 9.07          | 21.07                | 5.28      | <0.0002     | 0.007     | Selenomonas                                                           |
| 50Glu + 50Gln vs CO                             | 6.7           | −18.56               | 3.91      | <0.0003     | <0.0001   | Pelistega                                                             |
|                                                 | 9.07          | −21.16               | 5.28      | <0.0004     | 0.006     | Selenomonas                                                           |
| 25Glu + 75 Gln vs CO                            | 9.07          | −21.96               | 5.14      | <0.0005     | 0.004     | Selenomonas                                                           |
|                                                 | 740.85        | −3.7                 | 1         | <0.0006     | 0.023     | Blautia                                                              |
| 100Gln vs CO                                    | 34.79         | −20.86               | 3.03      | <0.0007     | <0.0001   | Fusobacterium                                                        |
|                                                 | 8.33          | −21                  | 3.12      | <0.0008     | <0.0001   | UBA1819, Family Ruminococcaceae                                      |
| Mixed additions vs Glu                          | 57.99         | 23.65                | 2.92      | <0.011      | <0.0001   | Pediococcus                                                          |
|                                                 | 11.38         | 21.04                | 3.41      | <0.0012     | <0.0001   | Enterococcus                                                         |
|                                                 | 1809.38       | 2.7                  | 0.7       | <0.0013     | 0.008     | Lactobacillus                                                        |
| Mixed additions vs Gln                          | 37.65         | 24.02                | 2.21      | <0.0009     | <0.0001   | Fusobacterium                                                        |
|                                                 | 59.71         | 9.67                 | 2.53      | <0.0010     | 0.013     | Pediococcus                                                          |
| **Day 21**                                      |               |                      |           |             |           |                                                                      |
| **Family level**                                 |               |                      |           |             |           |                                                                      |
| 100Glu vs CO                                    | 10.809        | 18.989               | 2.53      | <0.0001     | <0.0001   | Fibrobacteraceae                                                     |
| 75Glu + 25Gln vs CO                             | 10.809        | 18.689               | 2.59      | <0.0001     | <0.0001   | Fibrobacteraceae                                                     |
| 50Glu + 50Gln vs CO                             | 10.809        | 20.141               | 2.52      | <0.0001     | <0.0001   | Fibrobacteraceae                                                     |
| 25Glu + 75 Gln vs CO                            | 10.809        | 20.892               | 2.65      | <0.0001     | <0.0001   | Fibrobacteraceae                                                     |
| 100Gln vs CO                                    | 10.809        | 19.677               | 2.58      | <0.0001     | <0.0001   | Fibrobacteraceae                                                     |
|                                                 | 101.951       | 5.117                | 1.53      | 0.001       | 0.028     | Bacteroidales_RF16_group                                             |
|                                                 | 109.322       | 2.14                 | 0.68      | 0.002       | 0.037     | Peptococcaceae                                                       |
| **Genus level**                                 |               |                      |           |             |           |                                                                      |
| 100Glu vs CO                                    | 18.045        | 21.269               | 5.08      | <0.0001     | 0.006     | Selenomonas_3                                                        |
| 25Glu + 75Gln vs CO                             | 18.045        | 22.123               | 5.35      | <0.0001     | 0.007     | Selenomonas_3                                                        |
| CO vs AAs addition                              | 7.378         | −21.523              | 4.13      | <0.0001     | <0.0001   | Succiniclasticum                                                     |

Intestinal morphology. Table 4 shows the effect of dietary supplementation on the intestinal morphology measurements at d8 and d21. At d8, no difference due to diet was observed for villus height, villus width, crypt depth, and the number of goblet cells and duodenal glands. However, at d8, in the duodenum, the number of duodenal glands was higher in the 100Gln group as compared with the other groups supplemented with mixed doses of AAs ($P = 0.048$); a quadratic effect of the Glu:Gln ratio was observed ($P = 0.048$). At d8, in the jejunum, the number of goblet cells was lower in the CO group as compared with the 50Glu + 50Gln group ($P = 0.05$); in the ileum, the villus height tended to be higher in the 100Glu group as compared with the other groups supplemented with mixed doses of AAs ($P = 0.097$). The villus width tended to be higher in the CO group as compared with the 100Glu group ($P = 0.079$) and the number of goblet cells tended to increase linearly with the Gln dose ($P = 0.090$). No difference due to diet was observed for the interstitial score in any of the intestinal segments at d8. Diet influenced the intraepithelial score in the jejunum at d8; the CO group had a higher probability of having a higher intraepithelial lymphocyte score as compared with the groups supplemented with Glu, Gln or both ($P < 0.016$), and the 25Glu + 75Gln group showed a lower probability of a high intraepithelial lymphocyte score.
as compared with the CO group (\(P = 0.05\)) (Fig. 3). At d21, no differences due to diet were observed for villus height, villus width, crypt depth, and the number of goblet cells and duodenal glands (Table 4). At d 21, in the duodenum, the crypt depth tended to be higher in the 100Gln group as compared with the other groups supplemented with mixed doses of AAs (\(P = 0.083\)). In the jejunum, the villus width tended to be higher in the CO group as compared with the 25Glu + 75Gln group (\(P = 0.081\)) at d21. No difference due to diet was observed for the interstitial and intraepithelial scores in any of the intestinal tracts at d21.

**Intestinal gene expression.**

No difference due to diet was observed for the expression of the Innate Immune Signal Transduction Adaptor (MyD88), Nuclear Factor Kappa B Subunit (NFKB2), Tumor Necrosis Factor (TNF), C-X-C Motif Chemokine Ligand 8 (IL8gene CXCL8), Occludin (OCLN), Tight Junction Protein 1 (ZO-1), Mucin 13, Cell Surface Associated (MUC13), Glutathione Peroxidase 2 (IL8;gene CXCL8), Occludin (OCLN), Tight Junction Protein 1 (ZO-1), Mucin 13, Cell Surface Associated (MUC13), Glutathione Peroxidase 2 (GPX-2) and Glutamate-Ammonia Ligase (GLUL) and Regenerating Family Member 3 Gamma (REG3G) in the jejunal mucosa of piglets. Some effects or trends for GLUL, ZO-1 and GPX2 were found with the orthogonal contrasts as shown in Table 5. The CO group tended to have a lower expression of GLUL as compared with the groups supplemented with AAs (\(P = 0.088\)). The expression of ZO-1 was higher in the 100Gln group as compared with the groups supplemented with mixed doses of AAs (\(P = 0.025\)); a linear tendency of the diet was observed (\(P = 0.079\)). The expression of GPX-2 tended to be higher in the 100Glu group as compared with the other groups supplemented with AAs (\(P = 0.086\)).
Figure 3. The effect of the diet on the cumulative probabilities of having each score for the intraepithelial lymphocytes in the jejunum of piglets at 8 days post-weaning. (A) Comparison between the CO group and the groups supplemented with AAs; (B) Cumulative probabilities of having each score in each dietary group. Diet: CO = standard diet; 100Glu = CO plus 6 kg/Ton Glu; 75Glu + 25Gln = CO plus 4.5 kg/Ton Glu and 1.5 kg/Ton Gln; 50Glu + 50Gln = CO plus 3 kg/Ton Glu plus 3 kg/Ton Gln; 25Glu + 75Gln = CO plus 1.5 kg/Ton Glu and 4.5 kg/Ton Gln; 100Gln = CO plus 6 kg/Ton Gln.

Table 5. Effect of the dietary supplementation (6 g/T) with glutamate and glutamine in the different ratios on the jejunal gene expression of piglets at 8 days post-weaning. Gene1: Glutamate-Ammonia Ligase (GLUL); Occludin (OCLN); Tight Junction Protein 1 (ZO-1); Glutathione Peroxidase 2 (GPX-2); Innate Immune Signal Transduction Adaptor (MyD88); Tumor Necrosis Factor (TNF); Mucin 13 Cell Surface Associated (MUC13); C-X-C Motif Chemokine Ligand 8 (IL8/CXCL8); Nuclear Factor kappa B Subunit 2 (NFKB2); Regenerating Family Member 3 Gamma (REG3G). Diet2 CO = standard diet; 100Glu = CO plus 6 kg/Ton Glu; 75Glu + 25Gln = CO plus 4.5 kg/Ton Glu and 1.5 kg/Ton Gln; 50Glu + 50Gln = CO plus 3 kg/Ton Glu plus 3 kg/Ton Gln; 25Glu + 75Gln = CO plus 1.5 kg/Ton Glu and 4.5 kg/Ton Gln; 100Gln = CO plus 6 kg/Ton Gln. Significant values are in bold.
Discussion

Supporting the integrity, functionality and morphology of the gut is a key strategy for sustaining piglets during the weaning transition period. The results obtained in the present study demonstrated that mixing doses of Glu and Gln can benefit gut health and the growth of post-weaning piglets during the first 2 weeks post-weaning, sustaining the thesis that these AAs play a crucial role in supporting and restoring the gut health of piglets.

The results suggested that both AAs were able to improve piglet growth during the more acute inflammatory period (until the second week post-weaning) as compared with the control diet, the piglets of which recovered in the third week post-weaning. The recovery of the control group could have been due to compensatory or “catch up” growth which occur when animals are supplied with sufficient nutrition after restricting feed or following adverse conditions, such as weaning. In fact, it is known that transient anorexia and gut inflammation can increase piglet morbidity in the first weeks post-weaning while, after 2 weeks, the gut can recover, and improve the digestion and absorption of nutrients, promoting piglet growth. The faster recovery of piglets supplemented with Glu and Gln could have been related to their functional roles regarding gut health which will be discussed later in detail. The supplementation of Gln was associated with more promising effects than that of Glu; in fact, a linear effect with higher values regarding the ADG and the G:F ratio for the groups receiving more Gln than Glu was observed considering the overall period. In the present study, it tended to improve the villus height. Finally, a beneficial interaction between Glu and Gln (50Glu + 50Gln) was observed in the small intestine mucosal morphological structure during inflammation. This result confirmed the previous hypotheses that Gln could contribute to regulating the expression of the TJ proteins, thereby conferring a beneficial effect on the post-weaning recovery of the intestinal mucosa.

The intestinal mucosal structure is closely connected to positive intestinal integrity and barrier function. The results obtained in the present study suggested that Gln could linearly increase the expression of ZO-1 in the jejunum, confirming the beneficial effect of Gln on Zonula Occludens (ZO) family members which, together with occludin, claudins, and actin, provide a scaffold for the assembly and regulation of the expression and distribution of the tight junction (TJ) complex in the intestinal mucosa. This result confirmed the previous hypotheses that Gln could contribute to regulating the expression of the TJ proteins, thereby conferring a beneficial effect on the mucosal barrier function (in vivo, post-weaning piglets and in vitro) . Glutamine can benefit the tight junction
proteins by means of several mechanisms: (1) a direct effect on the enterocytes, providing energy which helps to maintain the integrity of the epithelial barrier; (2) increasing the functional integrity of the mitochondria, via the activation of heat shock proteins (HSPs) expressed by enterocytes, particularly HSP72, and; (3) preventing apoptosis under heat stress by means of its regulation of the mTOR and p38 MAP kinase pathways.\textsuperscript{36} In fact, when there was Gln deficiency, dysfunctional mitochondria and the accumulation of mitochondrial ROS induced epithelial barrier defects and disrupted the tight junction proteins\textsuperscript{37}. In the present study, Gln also tended to reduce the expression of GPX2 in the jejunum. The GPX2 gene (also called GI-GPx) encodes the most expressed selenoprotein of the glutathione peroxidase family in the intestine which stimulates the reduction of hydrogen peroxide derived from inflammation in the gut, thereby protecting the cells against oxidative damage\textsuperscript{38}. In agreement with the present study, previous evidence from Zhang et al.\textsuperscript{39} showed that Gln reduced the expression of GPX2 protein in the jejunum of post-weaning piglets. In the present study, this effect in reducing the GPX2 expression in the gut did not result in a variation of Glutathione Peroxidase 1 (GPX-1) and Reactive Oxygen Metabolite (ROM) levels in the blood of piglets. However, it should be noted that the results of the gene expression are strictly related to the sampling time and the specific tissue; thus, they do not always agree with the pathway metabolites investigated in the peripheral blood. Furthermore, it appears that GPX2 is not only related to oxidative stress but is involved in counteracting inflammatory responses in the gut\textsuperscript{40} by means of the regulation of the NF-kB pathway\textsuperscript{38}.

It should be emphasised that, in the present study, supplementation with Glu and Gln and, in particular, the 25%Glu-75%Gln diet, reduced the probability of having intraepithelial lymphocytes (IELs) in the jejunal mucosa at d8. Intraepithelial lymphocytes are a T-cell subpopulation located above the basement membrane and in between the intestinal epithelial cells, below the intercellular tight junctions connecting the intestinal epithelial cells. Their main role is to kill infected epithelial cells and protect the intestine against pathogens. Previous studies have suggested modulation of the IELs by Glu and Gln as reported by Lobley et al.\textsuperscript{41}. Although the studies in the literature have reported an increase in IELs by Glu and Gln, it should be noted that they were assessing the effect of AAs using acute challenge models, such as sepsis or colitis, characterised by severe impairment of the barrier function and immunity of the gut\textsuperscript{42–44}. In the present study, the results of the faecal score and growth performance suggested that the piglets in the 25%Glu-75%Gln group were not under severe health impairment; thus, the result obtained for the IELs suggested a lower inflammatory status of these animals as compared with the animals in the other groups.

Results based on in vitro studies have suggested that AAs, including Glu and Gln, can be utilised by intestinal bacteria. Data have suggested that from 22 to 40% of Gln is utilised by bacteria in the jejunal and ileal tracts\textsuperscript{45}. The results obtained in the present study suggested that the AAs supplementation did not profoundly influence the microbial composition in the large intestine of weaned piglets; in fact, no differences in the alpha and beta diversity indices were found. Nevertheless, some minor differences in the abundance of some taxa were observed. After 1 week, the supplementation of Gln reduced the bacteria belonging to the Fusobacteriaceae family, which is known to include Glu and Gln-fermenting bacteria with some differences at the species level\textsuperscript{46}. At the same timepoint, the supplementation of Gln reduced the bacteria belonging to the Enterococcaceae and Lactobacillaceae families (mainly Pediococcus, Enterococcus and Lactobacillus genera) compared with the mixed doses of the AAs. Previous studies suggested that Lactobacillus species display glutaminase activity (responsible for the conversion of Gln to Glu)\textsuperscript{47,48}, which can explain the increase in Lactobacillus genus in the groups in which Gln was available; less clear pieces of evidence have been reported for Enterococcus and Pediococcus species which, however, are known to be able to utilize both Gln and Glu\textsuperscript{49,50}. In addition, it should be considered that both Gln and Glu play a key role in pH homeostasis and stationary phase survival of lactic acid bacteria which may have contributed to the different selection of these bacteria, however additional studies are needed to fully explain the present results.

After 3 weeks of dietary supplementation, the relative abundance of Fibrobacteraceae was promoted in all the groups supplemented with AAs. Fibrobacteraceae is a small common bacterial family capable of adhering to lignocellulosic fragments, degrading cellulose in the rumen\textsuperscript{49,52}, being present in the intestinal microbiota of piglets\textsuperscript{53,54}; this benefits the host by fermenting dietary fibre into short-chain fatty acids (SCFAs). At the genus level, the relative abundance of Selenomonas was reduced at d8 and increased (in a dose-dependent way) at d21 in the groups supplemented with AAs. Selenomonas is implicated in the fermentation of soluble carbohydrates\textsuperscript{55}; however, it has also been recognised as a proteolytic bacterium\textsuperscript{56}, capable of fermenting Glu and Gln\textsuperscript{56}.

The comparison between CO and the groups supplemented with the AAs showed a decrease in the control group of the genus Succinicielasticum which is known for its ability to ferment succinate and convert it into propionate\textsuperscript{57}.

Overall, the results obtained suggested that, after 3 weeks, the supplementation of AAs could favour the growth of fermenting AA bacteria in the large intestine without compromising the gut microbial ecosystem. The results obtained in the present study suggested that both Glu and Gln were able to improve piglet growth during the more acute inflammatory periods (until the second week post-weaning). Supplementation with Gln was more promising, especially related to better maintenance of the intestinal barrier integrity as the groups receiving more Gln had better results. A favourable effect of mixing the Glu and Gln was observed on the immune parameters, gut morphological structure and barrier function of the small intestine. These effects contributed to reducing the number of days with loose faeces and increasing the faecal consistency in piglets, mainly in the first period post-weaning. In conclusion, the present study suggested that supplementation with Glu and Gln at a ratio from 50% Glu-50% Gln to 25% Glu-75% Gln would maintain gut health and the growth of post-weaning piglets.
Material and methods

Animals, study design and sampling. The in vivo trial was approved by the Ethics Committee for Experiments on Animals of the University of Bologna, Italy and by the Italian Ministry of Health (Authorisation n. 503/2018-PR, released 2 July 2018 in compliance with art. 31 of the D.lgs. 26/2014), and complied with the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines. At weaning (d0), 120 piglets (24 ± 2 days of age; initial BW 6.96 kg ± 0.11 kg) were moved to the experimental facility of the University of Bologna. The in vivo trial was carried out in two consecutive batches of 60 piglets each.

The piglets were assigned to one of the six experimental diets (10 replicates per diet, 2 piglets/each replicate) based on the litter of origin and BW: (1) standard diet (CO); (2) CO plus 6 kg/Ton Glu (100Glu); (3) CO plus 4.5 kg/Ton Glu and 1.5 kg/Ton Gln (75Glu + 25Gln); (4) CO plus 3 kg/Ton Glu plus 3 kg/Ton Gln (50Glu + 50Gln); (5) CO plus 1.5 kg/Ton Glu and 4.5 kg/Ton Gln (25Glu + 75Gln); (6) CO plus 6 kg/Ton Gln (100Gln). The diets did not include antimicrobials, zinc oxide or growth promoters. To design the diets, the nutrient values were estimated using EvaPig® software (v. 1.4.0.1; INRA, Saint-Gilles, France). Amino acid supplementation was provided on-top. The diet composition and chemical composition in terms of AAs are reported in Supplementary Table 2.

The piglets were housed in cages with a mesh floor. Room temperature was kept controlled from 30 °C at the beginning of the trial to 25 °C at the end of the trial, with a 1 °C decrease every 3 days. The piglets had free access to feed and water throughout the experimental period; feed was ad libitum supplied in a dry feeder.

At d8 (acute phase) and d21 post-weaning (recovery phase), half of the pigs were slaughtered. The piglets were deeply anaesthetised with Pentothal Sodium* (10 mg/kg BW, MSD Animal Health S.r.l., MI, Italy) and sacrificed using an intracardiac injection of Tanax* (0.5 mL/ kg BW, MSD Animal Health S.r.l., MI, Italy) in compliance to the ARRIVE guidelines.

Blood analysis. A total of 19 haematological parameters (erythrocyte traits: RBCs, HGB, haematocrit (HCT), MCV, MCH, MCHC, red cell distribution width, RDW; leucocyte traits: white blood cell count, leucocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, (K/µL and %), and platelet traits: platelet count) were detected using laser impedimetric cytometry.

Microbial analysis. Total bacterial DNA for microbiota analysis was extracted from samples collected from the caeca using a FastDNA® Spin Kit for Soil (MP Biomedicals, LLC, Santa Ana, CA, USA). The quantity and quality of the DNA were evaluated using a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). Next Generation Sequencing (NGS) was carried out as reported by Luise et al. (2019).
the perivascular area; 2 = leukocyte infiltration (50–90) with signs of perivasculitis and collagen shrinkage, and 3 = severe infiltration of leukocytes (> 90) with areas of necrosis63.

**Gene expression analysis.** Total RNA was extracted using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) and treated to remove contaminating DNA using a TURBO DNA-free® DNA Removal Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the recommended protocol. A total of 1000 ng of RNA was then converted into complementary DNA using a High-Capacity RNA-to-cDNA® Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Duplex Real-Time PCR reactions contained 2 μl cDNA and 8 μl mix containing primers, probes (Supplementary Table 3) and 2X TaqMan Mastermix, and was run in triplicate on the Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) with the following thermocycler settings: 50 °C for 2 min, 95 °C for 2 min and 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Hydroxymethylbilane synthase (HMBS) was used as a housekeeping gene. QuantStudio Design and Analysis Software v2.5 (Thermo Fisher Scientific, Waltham, MA, USA) was used for determining the gene expression cycle threshold (Ct) values. For each sample, the Ct value of the HMBS gene was subtracted from the ΔCt value of all the samples (ΔΔCt). The expression of the target gene was given as fold change calculated by 2−ΔΔ(Ct).

**Bioinformatic and statistical analysis.** Statistical analysis was carried out using SAS version 9.3 (SAS Inst. Inc., Cary, NC, USA). The GLM procedure was used to fit the measurements carried on piglets with a linear model including batch, litter of origin, sex of piglets and diet. Orthogonal contrasts were used to assess the effect of Glu/Gln supplementation as follows: CO vs. AAAs addition: (CO vs. 100Glu, 75Glu+25Gln, 50Glu+50Gln, 25Glu+75Gln), Glu alone vs. mixed addition (100Glu vs. 75Glu+25Gln, 50Glu+50Gln, 25Glu+75Gln), Gln alone vs. mixed addition (100Glu vs. 75Glu+25Gln, 50Glu+50Gln, 25Glu+75Gln). In addition, the linear and quadratic effects of AAs supplementation were tested. Data regarding the faecal index were log-transformed before the statistical analysis.

Data regarding the intestinal evaluation of the intestinal mucosa, such as categorical response variables in numerical order, were assessed using the Generalised Linear Mixed Model (GLIMMIX) procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA), considering a multinomial distribution and calculating the cumulative probabilities of having each score for each experimental diet.

Microbiota analysis was carried out using the DADA2 pipeline64. Taxonomic categories were assigned using the Silva Database (release 138) as a reference65. Alpha (Shannon, Chao1 and InvSimpson indices) and Beta diversity (calculated as the Bray Curtis distance matrix), as well as the abundance of taxonomic categories, were analysed using the PhyloSeq67, Vegan68 and DESeq269 packages. The Alpha diversity indices were analysed using an ANOVA model (lm function) including batch, diet, time point (d8 and d21) and the interaction between diet and time-point as factors. Beta diversity was analysed using a PERMANOVA Adonis test model (adonis.test function) which included batch, diet, time point, and the interaction between diet and time-point as factors. The effects on Beta diversity were visualised using a Non-Metric Multidimensional Scaling (NMDS) approach (plot_ordination function). The analysis was then carried out in the dataset composed of the reference animals was then subtracted from the ΔCt value of all the samples (ΔΔCt). The expression of the target gene was given as fold change calculated by 2−ΔΔ(Ct).

**Ethics declarations.** The procedures complied with Italian law pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna, Italy and by the Italian Ministry of Health (Approval No. 503/2018 of 2 July, 2018). The study was carried out in compliance with the ARRIVE guidelines.

**Data availability** The raw reads obtained are publicly available at the NCBI Sequence Read Archive (SRA) under the project number PRJNA798542. The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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D.L.: Conceptualisation, Methodology, Formal analysis, Visualisation, Writing—Original draft preparation. F.C.: Methodology, Formal analysis. T.C.D.: Conceptualisation, Writing—Review & Editing. L.G.: Formal analysis. G.R.: Conceptualisation, Writing—Review & Editing. W.L.: Conceptualisation, Formal analysis, Supervision, Writing—Review & Editing. P.T.: Conceptualisation, Methodology, Funding acquisition, Supervision, Writing—Review & Editing.

Competing interests

The authors have read the Journal’s policy and have the following competing interests. Co-authors T.C.D and W.L. are associated with Metex Noovistago which partially financed the project and they provided support for experimental design. The authors D.L., F.C., L.G., G.R., P.B. and P.T. have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Additional information

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