



Research article

Aquaculture faecal waste generates different products during anaerobic digestion depending on nutrient composition

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ABSTRACT

The inclusion of carbohydrate-rich ingredients in aquafeeds has resulted in an increased fraction of undigested material, culminating in faecal waste enriched in unutilized nutrients containing carbon, nitrogen and phosphorus. This study explored the impact of faecal composition, as influenced by diet, on the products of anaerobic digestion with a focus on organic acids (OAs). The aim was to *in vitro* assess the potential of faeces as internal carbon for denitrification, promoting circularity in recirculating aquaculture systems. In this regard, settleable faeces originating from six diets (DDGS, Dried distillers' grains with solubles; HFM, Hydrolyzed feather meal; IM, Insect meal; SCP, Single-cell protein; SSM, Shrimp shell meal; SWP, Seaweed protein) fed to European seabass, were incubated for a 14-day period in anoxic batch reactors. Nutrient solubilization (chemical oxygen demand, total Kjeldhal nitrogen, total phosphorus) was measured over time, and the final yield of OA was studied in relation to prokaryotic community composition. Results showed that digestion of faecal waste with a high crude-protein-to-carbohydrate ratio leads to an increased amount of OA which is greatly dominated by acetate. Among them, SSM faeces exhibited the highest final OA yield, resulting from a continuous increase over time. Despite variations in OA quantity and profile, prokaryotic composition did not substantially differ among treatments at the end of the trial, with only the relative abundance of three genera varying significantly (*Anaerostignum*, *Bythopirellula*, *Mycobacterium*). Yet, lactate concentration positively correlated with several taxa (*Trichococcus*, *Oleispira*, *Defluviitaleaceae*, *Anaerocolumna*, and *Carboxyliverga*) and butyrate with *Anaerostignum*. Alongside, ammonia release was minimal for all treatments, while phosphorus dissolution did not correlate to the faecal phosphorus content but was rather a result of acidification due to OA production. Overall, considering that an optimal carbon source for denitrification should produce a high amount of end OAs (i.e. acetate) along with low dissolved nitrogen and phosphorus, this research suggests that faecal waste originating from certain carbohydrate-rich aquafeed ingredients can be suitable as internal carbon source for denitrification.

1. Introduction

To uphold the expansion of the aquaculture industry in an era of limited marine resources, a variety of ingredients have been employed in aquafeeds (Naylor et al., 2009). The nutritional value of such alternative protein sources is commonly lower compared to fish meal due to their unbalanced amino acid profile and high carbohydrate content (Glencross, 2016). Therefore, their dietary inclusion results often in poor digestibility and increased production of excreted solids in the form of faecal waste (Kokou and Fountoulaki, 2018; Schumann and Brinker, 2020).

The excreted solids are typically rich in organic matter and nutrients containing carbon (C), nitrogen (N) and phosphorus (P), and stringent disposal regulations have been implemented to minimize eutrophication of the environment (Directive, 2000/60/EC). However, recovery of unexploited resources does not only represent a legal obligation but also a window of opportunity since valorization of fish sludge can contribute to a circular economy (Di Costanzo et al., 2021). Even though aquaculture waste could be potentially used as fertilizer for agriculture (Ahuja et al., 2020) or aquaponics (Zhang et al., 2021), application of marine sludge in this regard is limited due to its high salinity. Thus, anaerobic digestion of faecal waste is seen as an alternative valorization

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method for the extraction of high-value products (Choudhury et al., 2023), such as organic acids (OAs) which have a wide range of downstream applications (Atasoy et al., 2018).

In aquaculture, OAs originating from fish faeces have been used as an internal C source for biological N removal (Aboutboul et al., 1995; Letelier-Gordo et al., 2020b; Suhr et al., 2015b), with denitrification in this context facilitating the concurrent elimination of two waste products, faecal carbon and nitrate. However, denitrification efficiency under these conditions is normally low (Letelier-Gordo et al., 2020b) because the OA levels obtained after faecal digestion are often limiting for bacteria (low C:N ratio). Nonetheless, the OA yield depends on the biodegradability of faecal carbon which essentially relates to the physical, biological and chemical characteristics of the sludge (Mirzoyan et al., 2008). Even though mechanical breakdown (e.g. Meriac et al., 2015) and bacterial inoculation of faeces (reviewed in Romero-Güiza et al., 2016) have been previously employed to improve faecal carbon bioavailability, faecal composition seems to be a main determinant of the digestion outcome (Letelier-Gordo et al., 2015; Meriac et al., 2014a). Since dietary composition has been found to exert a major effect on faecal composition (Antony Jesu Prabhu et al., 2019; Fountoulaki et al., 2022; Prakash et al., 2023; Syropoulou et al., 2024), it is hypothesized that diet impacts the bioavailability of faecal C for subsequent OA production. This interrelation becomes very relevant in view of the constantly emerging diet formulations which can result in solid waste products of unpredictable bioavailability.

As such, the present research aimed to explore the impact of various dietary ingredients on the products of anaerobic faecal digestion, focusing on the faecal C bioavailability measured as OA production. In addition to quantifying OA yields, OA profiles were analyzed, since denitrification efficiency may vary depending on the type of OAs produced (Elefsiniotis et al., 2004; Elefsiniotis and Wareham, 2007). Alongside, bioconversion of N and P was also studied in order to determine the side-products emerging throughout digestion. Finally, to gain some further insight into the biological processes occurring during digestion of the faeces obtained from the different diets, prokaryotic community structures were also examined.

2. Materials & methods

2.1. Experimental design

Six types of settleable faeces (Table 1) were obtained from a preceding feeding trial (Syropoulou et al., 2024) in which juvenile European seabass (*Dicentrarchus labrax*) were fed several experimental diets (Supplementary Table S1), all formulated to meet their nutritional requirements. Fish were stocked in individual tanks with independent settling units. After fish were acclimatized to the dietary treatment over a period of 14 days, faeces were collected overnight for 7 consecutive days. Faecal contamination with feed spill was prevented by immediate removal of any uneaten pellets from the experimental units. The collected material was pooled per tank and stored at -20°C until analysis.

Faecal materials were subjected to anaerobic digestion at a salinity of 31.8 ± 0.9 psu, without any added inoculum. The six treatment groups were tested in duplicate, with replicates originating from the same experimental phase of the feeding trial described in Syropoulou et al. (2024). Faeces were thawed and 19 ± 3 g organic matter (OM) were transferred into twelve 1 L Blue Cap ISO bottles which served as anoxic batch reactors during the trial. Reactors were tightly sealed with DURAN GL 45 screw caps with two connections each to attach one nitrogen gas inlet hose and one sampling outlet hose, respectively. Appropriate mixing of the faeces was ensured by continuous magnetic stirring at 250 rpm, while a constant temperature of approximately 23°C was maintained by placing bottles in a temperature-controlled water bath. Anoxic conditions were achieved by purging with nitrogen gas for 45 min before the start of the trial.

The trial lasted for 14 days and 50-mL samples were obtained every 24 h for the first two days (day0, 1 and 2) and every 48 h for the remaining duration of the trial (day4, 8, 10, 12, and 14). Additionally, two subsamples of 2 ml were obtained both on day0 and day14 for determining the organic acid (OA) composition and prokaryotic community composition, respectively. The latter were frozen and stored at -20°C until DNA was extracted. At all timepoints, temperature, pH and

Table 1

Proximate nutrient composition (g/kg OM) of the faeces produced by European seabass after being fed with different test diets over a 6-week experimental period. Modified from Syropoulou et al. (2024).

	DDGS	HFM	IM	SCP	SSM	SWP	SEM	P-value
Crude protein (CP)	159 ^a	267 ^c	184 ^{ab}	214 ^{abc}	234 ^{bc}	158 ^a	13	**
Crude fat	58	72	58	68	52	52	3	ns
Carbohydrates ^a	784 ^b	661 ^a	758 ^b	718 ^{ab}	714 ^{ab}	790 ^b	14	**
Starch	206 ^b	132 ^a	192 ^b	158 ^{ab}	111 ^a	127 ^a	11	**
Non-starch polysaccharides (NSP) ^b	314 ^b	241 ^a	248 ^a	234 ^a	225 ^a	314 ^b	12	**
Arabinosyl	60.3 ^b	50.6 ^{ab}	51.4 ^{ab}	47.0 ^{ab}	43.7 ^a	41.4 ^a	2.0	*
Fucosyl	1.67	2.02	1.78	2.02	1.7	1.88	0.06	ns
Galactosyl	23.0	28.2	26.3	25.8	25.0	23.6	0.6	ns
Glucosyl	89.1 ^b	55.7 ^a	62.6 ^a	56.8 ^a	58.0 ^a	86.7 ^b	4.4	**
Mannosyl	17.2 ^b	11.1 ^a	12.8 ^a	13.3 ^a	12.8 ^a	11.8 ^a	0.6	**
Rhamnosyl	1.83 ^a	2.11 ^a	1.97 ^a	3.19 ^a	1.79 ^a	23.9 ^b	2.4	***
Xylosyl	92.5 ^b	64.6 ^a	67.4 ^a	62.8 ^a	56.9 ^a	68.3 ^a	3.5	**
Uronyl	27.0 ^a	27.1 ^a	23.6 ^a	23.1 ^a	25.3 ^a	51.4 ^b	3.2	**
Ash	1044 ^a	1351 ^{bc}	1088 ^{ab}	1477 ^c	1367 ^{bc}	1272 ^{abc}	49	**
Total phosphorus (TP)	31 ^a	32 ^a	40 ^{ab}	54 ^b	71 ^c	34 ^a	4	***
Calcium (Ca)	63 ^a	86 ^b	85 ^b	99 ^b	187 ^c	85 ^{ab}	12	***
Iron (Fe)	1.3 ^a	1.8 ^b	1.6 ^{ab}	1.9 ^b	1.6 ^{ab}	3.2 ^c	0.2	***
Magnesium (Mg)	41 ^a	51 ^{bc}	44 ^{ab}	60 ^c	52 ^{bc}	47 ^{ab}	2	**
Manganese (Mn)	0.21 ^a	0.27 ^a	0.46 ^b	0.30 ^a	0.28 ^a	0.24 ^a	0.02	***
Zinc (Zn)	0.31 ^a	0.43 ^{ab}	0.45 ^{ab}	0.50 ^b	0.48 ^b	0.38 ^{ab}	0.02	*
COD:TP:TKN (g/g) ^c	10:1.2:1 ^b	6:0.8:1 ^{ab}	12:1.4:1 ^b	8:1.6:1 ^{ab}	8:1.9:1 ^a	8:1.3:1 ^{ab}	Na	*

DDGS, Dried distillers' grains with solubles (wheat); HFM, Hydrolyzed feather meal; IM, Insect meal; SCP, Single-cell protein; SSM, Shrimp shell meal; SWP, Seaweed protein; Na; not applicable.

Values are shown as means (n = 2) with a standard error of the mean (SEM); values lacking a common superscript are significantly different. P-value: >0.05, ns; *, <0.05, **, <0.01, ***, <0.001.

^a Calculated as: OM – CP – Crude fat.

^b Calculated as the sum of all analyzed NSP constituents.

^c Calculated as: Chemical Oxygen Demand (COD)/Total phosphorus/Kjeldahl Nitrogen.

O₂ were measured using a handheld instrument (WTW multi 340i GmbH, Weilheim, Germany).

2.2. Chemical analyses

Test kits (LCK365, Hach Lange, Germany) were used for the quantification of OAs in fresh samples which were priorly centrifuged (4500 rpm at 4 °C for 15 min) and filtered (0.45 µm). The content of individual OAs (lactate, formate, acetate, propionate, butyrate) was measured in raw (uncentrifuged), pretreated (clarified with Carrez reagent) samples by high performance liquid chromatography using a HPLC Shimadzu instrument equipped with an Aminex HPX 87H column (300 × 7.8 mm BIO-RAD) at 45 °C (Shimadzu, model CTO-30A) and a UV-Vis detector (Shimadzu, model SPD-10AV) at a wavelength of 210 nm. For the mobile phase, sulfuric acid (H₂SO₄; 5 mmol L⁻¹) was used as an eluent at a flow rate of 0.6 mL min⁻¹ to which 30 mM of crotonate was added as internal standard. The selected OAs were quantified using individual standard curves; each curve included 7 standards (plus 0; Sigma-Aldrich, Germany) ranging from 0.031 to 2 g L⁻¹ for acetic acid, from 0.0156 to 1 g L⁻¹ for propionic acid, and from 0.0078 to 0.5 g L⁻¹ for formic acid, butyric acid, and lactic acid.

For the other chemical analyses, samples were immediately centrifuged (4500 rpm at 4 °C for 15 min), filtered (0.45 µm), fixed with 1% v/v H₂SO₄, and stored at -20 °C upon analysis. Soluble chemical oxygen demand (sCOD) was determined using digestion vials (LCK1914, Hach Lange, Germany). The analytical subsamples for total ammonia nitrogen (TAN) and phosphorus (P-PO₄³⁻) were analyzed with an autoanalyzer (SAN Plus, Skalar, Breda, The Netherlands; Skalar protocol number. 155-006 w/r; Orthophosphate - Stannous Chloride Method).

2.3. Prokaryotic analysis

Before DNA extraction, samples were centrifuged at 10,000 g for 1 min and supernatant was subsequently removed. DNA was extracted using the DNeasy PowerLyzer™PowerSoil DNA Isolation Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA concentration was measured with NanoDrop (Thermo Fisher Scientific, US). Library preparation was performed targeting the V4 region of the 16S rRNA gene, which was amplified with primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011). The polymerase chain reaction (PCR) products were sequenced using a MiSeq PE250 Next Generation system (Illumina) by Genome Quebec, following the company's protocol. Blank samples without DNA template were used as controls. Sequences have been submitted to NCBI Sequence Read Archive database under the study accession code PRJNA1120889.

Analysis of the 16S rRNA gene reads was done using the DADA2 Pipeline (1.8) (Callahan et al., 2016). The SILVA reference database was used for the annotation of the Amplicon Sequence Variants (ASVs) (McLaren, 2020). A rooted phylogenetic tree was constructed using qiime2 (Bolyen et al., 2019). Downstream analysis was performed using the R microeco package (v1.4.0). The dataset was rarified at the threshold of 11,366 reads to achieve a common sequencing depth for all samples (Supplementary Fig. S1). Alpha diversity was measured with the Phylogenetic Diversity (PD) index, and beta diversity was assessed by Principal coordinate analysis (PCoA) analysis based on weighted UniFrac distance metrics.

2.4. Statistical analysis

All data were analyzed with R software (R Core Team, 2022; version 4.2.1) and are reported as means of two replicates (n = 2) with a standard error (SE) or a standard error of the mean (SEM). The confidence level for all analyses was set at 95%. Pairwise correlations between variables were analyzed with either Pearson or Spearman's rank correlation.

To investigate the effect of dietary treatment (i.e. type of faecal waste) on nutrients, a repeated measures analysis was performed to test the fixed effect of diet, time and their interaction effect. For TAN and P-PO₄³⁻, a mixed model for repeated measures was used instead due to the unbalanced experimental design that was created after removal of outliers induced by analytical errors (Supplementary Fig. S2). In all cases, when a significant effect of diet by time interaction was found, the effect of diet was investigated separately for the last time point (day14) using a one-way ANOVA followed by a Tukey honest significant difference (HSD) test when significant. When normality was not confirmed with a Shapiro-Wilk test even after data transformation, an Aligned ranks transform ANOVA was applied instead for models with diet, time and their interaction as fixed factors. When significance was found, a subsequent non-parametric analysis with Kruskal-Wallis test was performed to investigate the effect of diet exclusively at day14. Differences in the final OA composition among the dietary treatments were tested with permutational multivariate ANOVA (PERMANOVA), whereas differences in individual OAs at day14 were analyzed with ANOVA.

The impact of diet on alpha diversity was tested with repeated measures analysis for the fixed effect of diet, time and their interaction. Similarly, the factors affecting prokaryotic community dissimilarity were studied using a two-way PERMANOVA. Linear discriminant analysis Effect Size (LEfSe) and Metastats were used to identify differentially abundant taxa among diets and between time points. Redundancy analysis (RDA) and Pearson correlation analysis were employed to explore the relationship between relative abundances of taxa in the digested faecal waste and faecal nutrients (e.g. OAs, COD, TKN, TP).

3. Results

3.1. Carbon hydrolysis and fermentation

The pH decreased significantly over time, with the rate of decline varying by diet (p_{diet}<0.05, p_{time}<0.001, p_{diet x time}<0.001). Across all treatments, a drastic pH drop was observed on day1, followed by an apparent stabilization. On day14, pH values ranged between 4.8 and 6.4 but were not statistically different among dietary treatments (Fig. 1A; p > 0.05). Although, carbon solubilization (sCOD/OM) increased with time and differences among diets became larger (Fig. 1B; p_{diet x time}<0.05), differences in final sCOD/OM values were marginally insignificant (p = 0.05032). Organic acids (OAs) correlated positively with sCOD (p < 0.001, r_s = 0.826), exhibiting also an increase over time (p_{time}<0.05). Differences in OA/OM among diets became more pronounced over time (p_{diet x time}<0.05); after a steep rise on day1, OA production either stagnated (SWP, DDGS), increased slightly (IM, SCP, HFM) or increased markedly (SSM), resulting in a higher OA yield for SSM on day14 (Fig. 1C; p < 0.001). Although OA composition, as found on day14, differed overall among treatments (p < 0.001), it was always dominated by acetate (Fig. 1D). Accordingly, faeces obtained from fish fed with SSM resulted in the highest absolute amounts of acetate and propionate (Supplementary Table S3). Other OAs varied among diets, with butyrate being consistently present but higher in HFM, lactate being detected only in DDGS, and formate levels being similar across treatments.

3.2. Nitrogen and phosphorus dissolution

Total ammonia nitrogen (TAN/OM) decreased within two days for all treatments but increased back to the initial levels over the course of the trial and seemed to remain stable after the initial drop (Fig. 2A; p_{diet}<0.05). Although fluctuations in TAN/OM varied by diet (p_{diet x time}<0.001), final differences among treatments seemed to reflect the initial status, with SCP yielding the highest levels of TAN/OM on day14 (p < 0.01). Similarly, nitrogen dissolution in relation to the nitrogen content of the faecal waste (TAN/TKN) appeared to stabilize from day8 onwards, following variable fluctuations depending on the diet

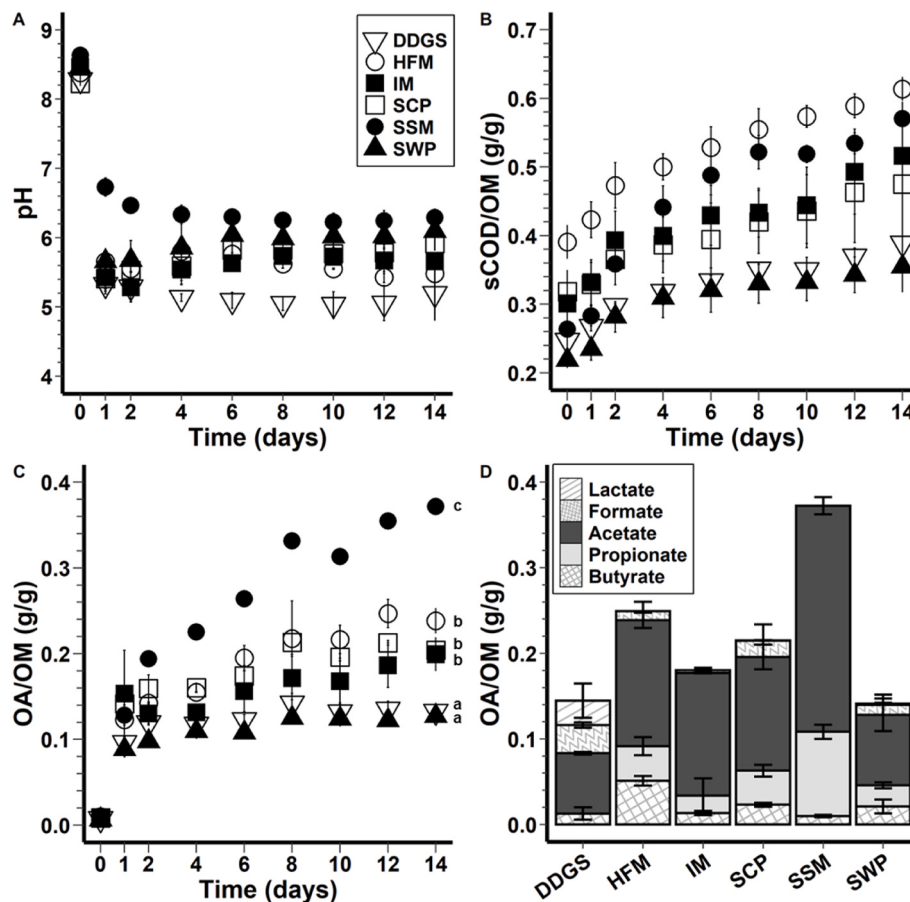


Fig. 1. Hydrolysis and fermentation profiles after anaerobic digestion of faecal waste produced by European seabass: A) pH profile, B) Soluble carbon expressed as chemical oxygen demand (sCOD) per unit of organic matter (OM), C) Organic acid (OA) production per unit of OM, and D) OA composition per unit of OM as found on day14. Values are shown as means ($n = 2$) with a standard error (SE); values lacking a common superscript are significantly different on day14 ($p < 0.05$). DDGS, Dried distillers' grains with solubles (wheat); HFM, Hydrolyzed feather meal; IM, Insect meal; SCP, Single-cell protein; SSM, Shrimp shell meal; SWP, Seaweed protein.

($p_{\text{diet}} < 0.05$, $p_{\text{time}} < 0.001$, $p_{\text{diet} \times \text{time}} < 0.01$), with DDGS resulting in the highest amount of TAN release on day14 (Fig. 2B, $p < 0.01$). Phosphorus dissolution ($\text{P-PO}_4^{3-}/\text{OM}$) was negatively correlated with pH ($p < 0.001$, $r_s = -0.356$), and after a rapid increase over day1, it leveled off ($p_{\text{time}} < 0.001$). Despite differences developing among diets over time ($p_{\text{diet} \times \text{time}} < 0.001$), $\text{P-PO}_4^{3-}/\text{OM}$ levels were similar among treatments at the end of the trial (Fig. 2C, $p > 0.05$). When phosphorus dissolution was studied in relation to the phosphorus content of the faecal waste ($p_{\text{diet}} < 0.001$, $p_{\text{time}} < 0.001$, $p_{\text{diet} \times \text{time}} < 0.001$), DDGS and SSM yielded the highest amount of $\text{P-PO}_4^{3-}/\text{OM}$ on day14 (Fig. 2D, $p < 0.01$).

3.3. Microbial diversity and community composition

Alpha diversity of the prokaryotic community quantified by the Phylogenetic Diversity (PD) index (Fig. 3A) decreased over time similarly for all diets ($p_{\text{diet}} > 0.5$, $p_{\text{time}} < 0.001$, $p_{\text{diet} \times \text{time}} > 0.05$). Likewise, beta diversity based on weighted UniFrac distances (Fig. 3B) differed from the start to the end of the trial but was unaffected by diet ($p_{\text{diet}} > 0.5$, $p_{\text{time}} = 0.001$, $p_{\text{diet} \times \text{time}} > 0.05$). LEfSe analysis identified 46 genera contributing to community differences between the two time points, with *Vibrio* and *Psychrobacter* having the highest Linear Discriminant Analysis (LDA) scores (both > 5). The relative abundance of 245 genera differed between day0 and day14, and only eight genera were present at both time points when considering the most dominant taxa (Fig. 3C). Despite the insignificant effect of diet on the prokaryotic composition after anaerobic digestion ($p > 0.05$), pairwise comparisons on day14 revealed some significant differences in the relative

abundances of individual taxa (Supplementary Table S3), alongside various insignificant ones (Supplementary Fig. S3).

The relative abundance of certain bacterial groups in the digested faeces (*Carboxyliverga*, *Anaerocolumna*, *Deftuviitaleaceae* UCF-011, *Oleispira*, *Trichococcus*, and *Anaerostignum*) correlated with the final yield of the different OAs tested (Fig. 4A). After studying the relationship between the prokaryotes found at day14 and the faecal nutrients (COD, TKN, TP) provided to them on day0, *Clostridium sensu stricto* 7 was the only one correlating with the COD content of the faeces ($p < 0.05$ $r_p = 0.890$). The origin of faecal COD and whether this was derived from carbohydrate, crude protein, or fat was not found to correlate with the relative abundance of any genus. However, COD derived from fat was found to correlate with the prokaryotic composition as a whole ($p < 0.05$; Fig. 4B).

4. Discussion

The present research aimed to explore the impact of faecal composition, as influenced by dietary ingredient inclusion, on the products of anaerobic digestion. Since conditions of high salinity (Choudhury et al., 2022) and low pH (Atasoy et al., 2018) inhibit methanogenesis, digestion products in this study originated predominantly from hydrolysis and acidogenesis (i.e. fermentation). However, pH is also known to affect hydrolysis (Gomec and Speece, 2003), and fermentation can lower pH levels due to organic acid accumulation. Although this drop in pH from acidogenesis likely reduced hydrolysis yields, buffering agents such as ammonia and calcium maintained pH levels above 5 (Fig. 1A).

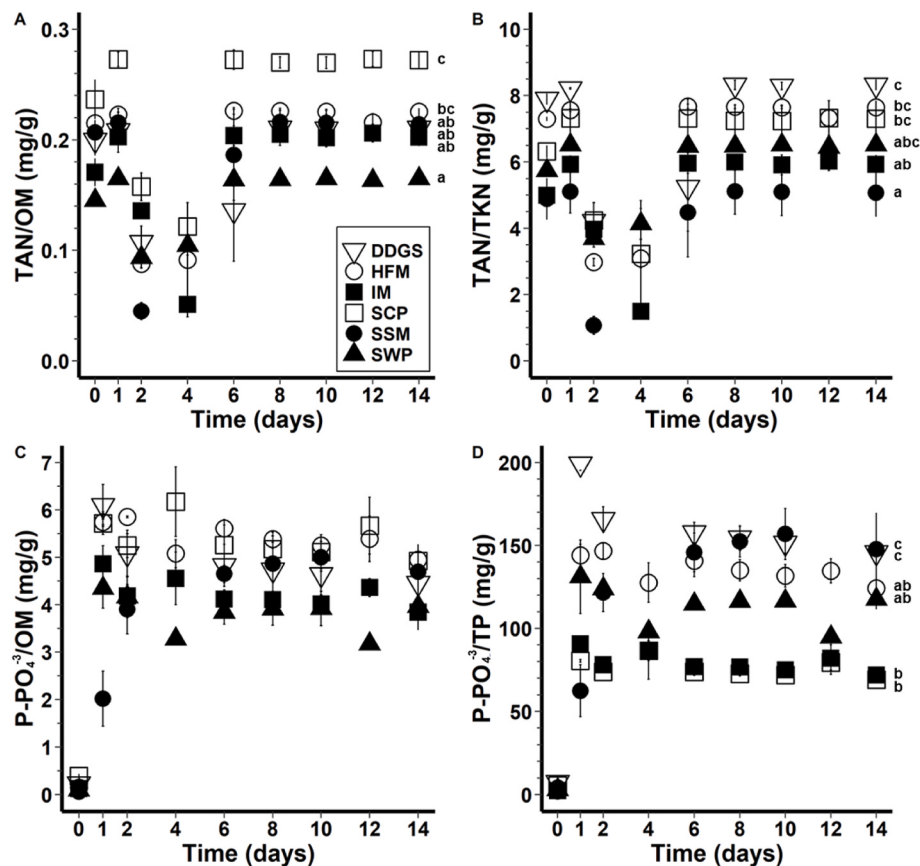


Fig. 2. Solubilized nutrients during anaerobic digestion of faecal waste produced by European seabass: A) Total ammonia nitrogen (TAN) per unit of organic matter (OM), B) TAN per unit of total Kjeldhal nitrogen (TKN), C) Phosphorous as orthophosphate ($P\text{-PO}_4^{3-}$) per unit of OM, and D) $P\text{-PO}_4^{3-}$ per unit of total phosphorus (TP). Values are shown as means ($n = 2$) with a standard error (SE); values lacking a common superscript are different on day14 ($p < 0.05$). Values for DDGS and SSM as obtained for day4 & 12 (not shown) were considered outliers and were therefore excluded from the statistical analysis. DDGS, Dried distillers' grains with solubles (wheat); HFM, Hydrolyzed feather meal; IM, Insect meal; SCP, Single-cell protein; SSM, Shrimp shell meal; SWP, Seaweed protein.

This effect was particularly evident for SSM faeces, which maintained a higher overall pH compared to the other treatments, likely due to their higher calcium content. Nonetheless, the pH conditions across all treatments allowed for the establishment of hydrolytic and fermentative microbial communities (Mirzoyan et al., 2010). These communities were unaffected by faecal substrate variations, contrary to prior findings (Dargode et al., 2022), with few exceptions among individual taxa (Supplementary Table S3). As such, anaerobic digestion effectively enabled organic matter (OM) degradation and thus conversion of particulate nutrients into soluble equivalents across all treatments.

Anaerobic digestion of faecal waste facilitated carbon solubilization, as evidenced by the net gain in soluble chemical oxygen demand (sCOD). The final yield of sCOD was generally higher for faeces with a higher crude-protein-to-carbohydrate ratio (HFM, SSM, IM), though this difference was not statistically significant (Fig. 1B). The elevated sCOD yield of these treatments was influenced by both higher initial sCOD values and increased sCOD production due to digestion ($\Delta\text{sCOD} = \text{sCOD}_{\text{day14}} - \text{sCOD}_{\text{day0}}$). This finding is in contrast to existing literature, which suggests that faeces with a lower protein-to-carbohydrate ratio (e.g. DDGS, SWP) generate higher amounts of sCOD (Letelier-Gordo et al., 2015). The discrepancy might be explained by the likely high lignin and cellulose content in DDGS and SWP, respectively. Despite an apparent increase in the relative abundance of candidate lignin- and cellulose-degrading bacteria, like *Pseudoalteromonas* (Sun et al., 2023) and *Sunxiuqinia* (Dai et al., 2016) for DDGS and SWP respectively, carbon solubilization remained low in these treatments. This confirms that such carbohydrates are highly resistant to degradation even after prolonged digestion periods (Meriac et al., 2014b; van Rijn et al., 1995).

Alongside sCOD, organic acids (OAs) also increased which indicates that part of the hydrolyzed carbon was further fermented. Since carbohydrates were the major component of all faecal materials (>66% OM), OA production primarily originated from carbohydrate fermentation, making OA/OM variations a useful indicator of carbohydrate degradability over time. Despite differences in carbohydrate degradability across treatments, OA production in all reactors seemed to decline exponentially, with the highest fermentation rates being observed at the onset of the digestion period (Fig. 1C), corroborating former findings (Soares et al., 2010; Suhr et al., 2015a). Despite the strong positive correlation between sCOD and OA, SSM exhibited the highest OA production surpassing HFM by 56.3% in the final OA yield (Fig. 1C), even though the latter had a slightly higher final sCOD yield. This suggests that fermentability and thus bioavailability of carbon may vary among faecal materials, with those yielding more OAs, like SSM, being likely more promising as carbon sources for denitrification, since OAs are generally preferred electron donors compared to more complex molecules like carbohydrates and proteins (Gao et al., 2020a,b). The vastly lower OA/sCOD ratio obtained for DDGS and SWP on day14 (0.34 and 0.36 respectively) compared to SSM (0.65) might additionally be attributed to carbon fermentation into other products besides OAs, such as alcohols, which are commonly produced during carbohydrate fermentation (Letelier-Gordo et al., 2017). Even though alcohols were not presently measured, their quantification should be considered in future studies.

The effect of faecal composition on fermentation is reflected by differences in the final OA profile (Fig. 1D; Table S2). Even though its levels were higher for SSM, acetate was the major OA component regardless of

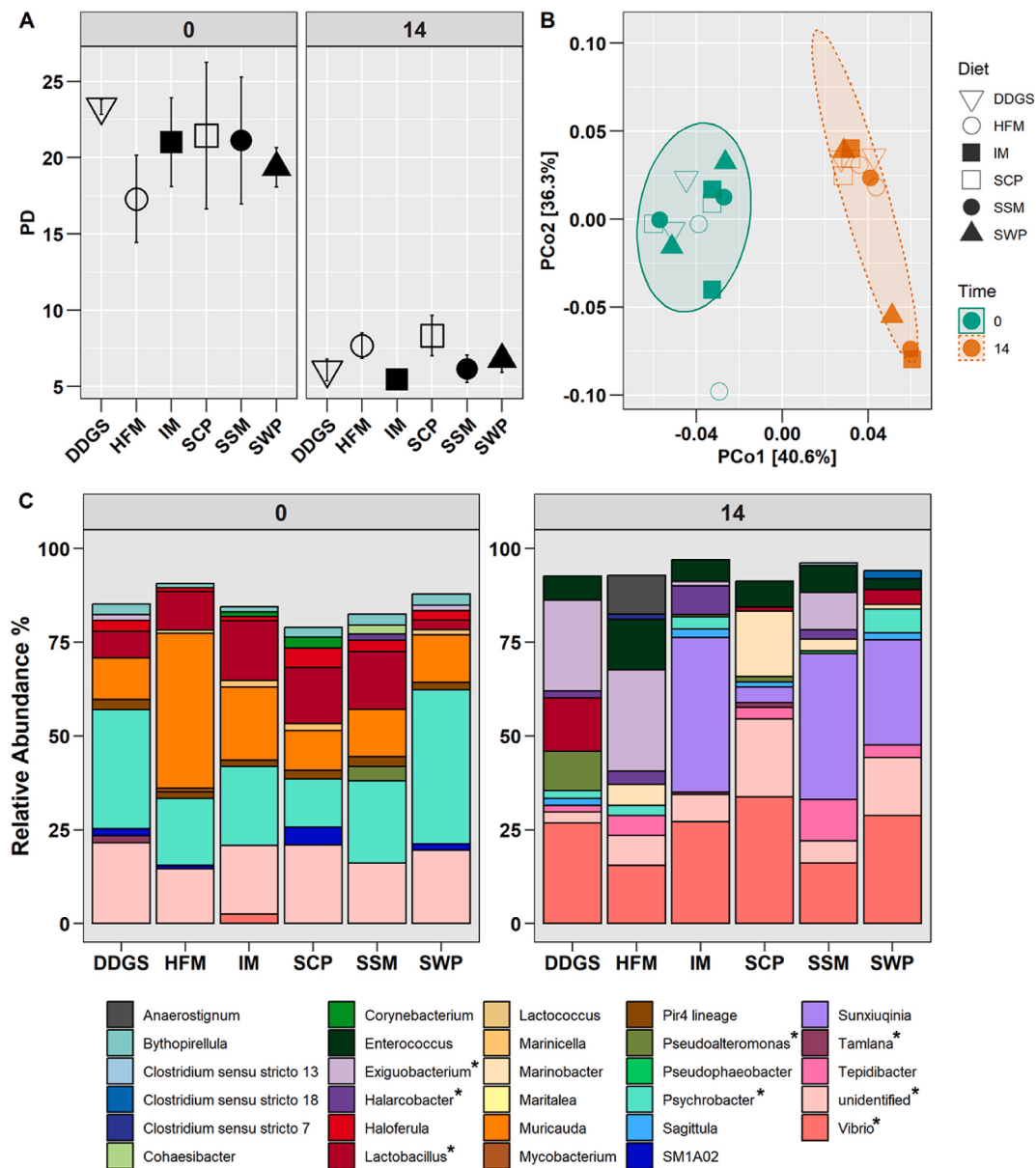


Fig. 3. Prokaryotic community structure before and after anaerobic digestion of faecal waste produced by European seabass: A) Prokaryotic diversity at day0 and day14 based on the phylogenetic diversity index (PD), B) Prokaryotic composition dissimilarity shown by Principal coordinate analysis (PcoA) analysis based on weighted UniFrac distance metrics, C) Relative abundances of the 10 most abundant genera at day0 and day14, with asterisks indicating taxa represented at both time points. DDGS, Dried distillers' grains with solubles (wheat); HFM, Hydrolyzed feather meal; IM, Insect meal; SCP, Single-cell protein; SSM, Shrimp shell meal; SWP, Seaweed protein.

the treatment, since it can be produced through several pathways and by any organic substrate (Batstone et al., 2002). The presence of intermediate OAs (lactate, formate, propionate, butyrate) is indicative of an incomplete anaerobic digestion, with the low pH observed (Fig. 1A) potentially hindering their further conversion into acetate, as previously argued (Letelier-Gordo et al., 2017). High levels of intermediate OAs in materials like DDGS and SWP imply that these sources might be less effective for denitrification, since intermediate OAs generally lead to lower denitrification rates compared to acetate (Elefsiniotis et al., 2004; Elefsiniotis and Wareham, 2007).

Lactate formation was observed only for DDGS, as expected due to its high carbohydrate content and previous findings linking carbohydrate fermentation to elevated lactate levels (Leenhouwers et al., 2008; Lü et al., 2021). This was confirmed by the higher relative abundance of typical lactic-acid-producing bacteria, such as *Lactobacillus* and *Trichococcus* (Holzapfel and Wood, 2014), observed for DDGS on day14

(Fig. 3C, Supplementary Fig. S3A). Although these differences were not statistically significant due to the low number of replicates and high variation, the observed trend aligns with expected microbial community shifts, suggesting that larger sample sizes may be needed to substantiate these findings. The positive correlation between *Trichococcus* and lactate further suggests its involvement in lactate production. While more bacterial groups (*Carboxylicivirga*, *Anaerocolumna*, *Deftuviitaleaceae UCG-011*, *Oleispira*) were here identified as potential contributors to lactate production, there is no previous record of their involvement in this metabolic pathway. However, other members of *Deftuviitaleaceae* family have been found to produce lactate (Ma et al., 2017a,b).

Faeces with higher crude protein content (SSM, HFM) exhibited the highest propionate yields, consistent with previous studies that found a propensity for propionate production for protein-rich substrates (Ma et al., 2017a,b). Although butyrate has been formerly associated with carbohydrate fermentation, of both starch (Leenhouwers et al., 2008)

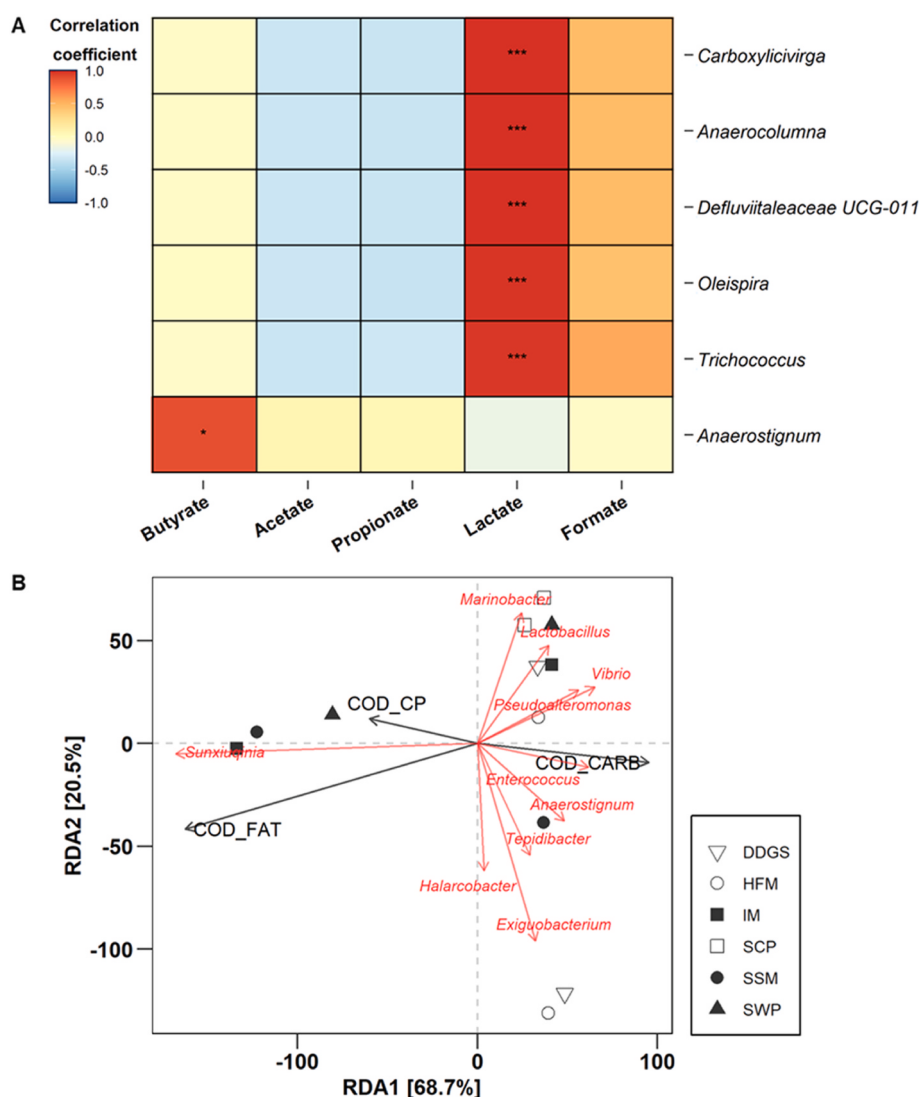


Fig. 4. Relationships between the final prokaryotic community structure, measured as relative abundances, and faecal nutrients (g/g organic matter). A) Correlation plot demonstrating bacterial genera that show significant correlations with organic acids at day14, and B) Redundancy analysis (RDA) ordination plot illustrating the relationship of prokaryotic communities (scatter points) and dominant prokaryotic genera (red arrows) with the provided faecal chemical oxygen demand (COD; black arrows) deriving from carbohydrate (COD_CARB), crude protein (COD_CP), or fat (COD_FAT). DDGS, Dried distillers' grains with solubles (wheat); HFM, Hydrolyzed feather meal; IM, Insect meal; SCP, Single-cell protein; SSM, Shrimp shell meal; SWP, Seaweed protein. P-value: >0.05, ns; *, <0.05; **, <0.01; ***, <0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and non-starch polysaccharide (NSP) type (Yu et al., 2022), in the present study the highest butyrate production was found in the faeces with the highest crude-protein-to-carbohydrate ratio (HFM) which suggests that butyrate production from amino acids (Batstone et al., 2002) may be considerable. The higher relative abundance of *Anaerostignum* for HFM on day14 (Fig. 3C, Supplementary Table S3), a genus reported to assist protein fermentation (Ueki et al., 2017), further suggests that protein fermentation is a notable contributor to butyrate production (Jackson et al., 2024).

Anaerobic digestion of faecal waste enhanced the solubilization of other nutrients besides carbon, namely phosphorus and nitrogen. While previous studies suggest that the inadvertent increase of dissolved nitrogen and phosphorus during faecal digestion renders internal carbon an unsuitable source for denitrification in RAS (Conroy and Couturier, 2010), this study found minimal nitrogen release across all treatments. Final TAN levels were similar to the initial ones, contrasting with former studies where nitrogen solubilization amounted for 14–36% of TKN (Letelier-Gordo et al., 2015; Suhr et al., 2015a). This discrepancy might be related to the lower faecal protein content in the present study, which

ranged from ~16 to 27% organic matter (OM) compared to the range of ~26–43% OM reported by Letelier-Gordo et al. (2015). On this basis, the reduced protein content implies lower nitrogen availability for solubilization. As a result, the limited TAN release was likely due to extensive bacterial uptake, particularly evident from the steep decline in TAN early in the trial. Although nitrogen assimilation by bacteria likely exceeded nitrogen solubilization initially (Kirchman, 1994), the subsequent increase in TAN suggests a stabilization of bacterial activity and a shift towards higher nitrogen solubilization. Thus, faecal materials with a low protein content may lead to negligible ammonia net yields, as the solubilized nitrogen is predominantly utilized by bacteria.

Contrary to the marginal production of dissolved nitrogen, phosphorus as orthophosphate increased considerably during anaerobic digestion (Fig. 2C), with the order of increase (up to ~15% of total phosphorus) being similar (Letelier-Gordo et al., 2015) or lower (Suhr et al., 2015a) to previously reported values. The concentration of dissolved phosphorus was inversely related to pH, as phosphorus solubilization is postulated to be a consequence of acidification (Conroy and Couturier, 2010). In the present study, the drop in pH due to OA

production corresponded to immediate phosphorus dissolution. This was exceptionally high for DDGS (Fig. 2D) possibly due to phytase residues originating from the dietary ingredient processing that further enhanced phosphorus bioavailability and thus dissolution. Despite differences in phosphorus levels among faecal waste (Table 1), the final P-PO_4^{3-} yield was similar for all treatments, indicating no apparent relationship between phosphorus dissolution and faecal total phosphorus (TP) content. This could be due to solubilized phosphorus reacting with other minerals to form precipitates (Yogev et al., 2020). For instance, while the high TP content in SSM faeces may have initially resulted in elevated dissolved phosphorus levels, the abundant calcium might have caused phosphorus to precipitate as calcium phosphate, thereby lowering the net P-PO_4^{3-} concentration. In phosphorus cycling, the contribution of P-PO_4^{3-} uptake by bacteria is considered negligible for phosphorus cycling in the reactors since the amount required for their growth is low (Hamza et al., 2019). This might explain why no prokaryotic taxon correlated with TP levels in the faecal substrate.

Finally, another concern, particularly with the digestion of marine sludge, is the emission of hydrogen sulfide (H_2S) (Letelier-Gordo et al., 2020a). Hydrogen sulfide, generated during the anaerobic degradation of organic matter, presents a challenge in wastewater treatment due to its corrosive nature, unpleasant odor (Jiang et al., 2015), and high toxicity which threatens fish health in RAS (Lazado et al., 2024). Although H_2S levels were not measured in this study, the phylum Desulphobacterota, containing many known sulfate reducers, emerged as the eighth most dominant after anaerobic digestion. However, its relative abundance was not higher for diets containing sulphonated polysaccharides like SWP, as expected (Supplementary Fig. S3B). Similarly, the relative abundance of other bacteria associated with sulfide production, such as Peptostreptococcaceae (Letelier-Gordo et al., 2020a) (Supplementary Fig. S3C), was consistent across all treatments. Although a higher abundance of sulfate-reducing bacteria could indicate a greater risk for H_2S production, lack of variation in these populations among treatments suggests that faecal composition may not immediately affect H_2S production after anaerobic digestion.

5. Conclusion

The present findings indicate that faecal composition, influenced by dietary ingredient inclusion, affects the products of anaerobic digestion. Overall, faecal waste with higher crude-protein-to-carbohydrate ratios produces a greater amount of organic acids and specifically, higher acetate levels. While organic acid production is faster at the onset of digestion regardless of faecal composition, prolonged digestion yields considerably higher organic acid levels for certain materials (e.g. SSM) but not for those containing complex carbohydrates. Ammonia release is marginal when faecal crude protein content is low (<27% organic matter), while phosphorus release is substantial and unrelated to faecal phosphorus content. Considering that an optimal carbon source for denitrification is one that produces the highest amount of end organic acids (i.e. acetate) and the lowest dissolved nitrogen and phosphorus, this research suggests that aquafeeds including certain carbohydrate-rich ingredients can produce faeces suitable as an internal carbon source for denitrification, while providing protein-sparing benefits for the fish host. These results may therefore assist aquafeed formulators in creating RAS feeds that benefit both the fish and the system. However, further research on the degradability and fermentability of individual carbohydrate types is recommended to gain deeper insights into the microbial utilization of carbohydrates during anaerobic digestion.

CRediT authorship contribution statement

Elisavet Syropoulou: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Detmer Sipkema:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition,

Conceptualization. **Sophie Elise Smit:** Writing – review & editing, Investigation. **Johan W. Schrama:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Fotini Kokou:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2024.122826>.

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