Metallurgical and Materials Engineering *Research paper*

Incurred Changes in Water and Fat-Soluble Vitamin Profile of Alternative Ingredient *Spirodela polyrhiza* Based Partial Fishmeal Substituted Aquafeed: Effect of Long-Duration Variable Temperature Storage

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Abstract: The study aims to evaluate effects of storage duration and storage temperature on changes in vitamin profile of formulated aquafeeds. Two aquafeeds are formulated one as extrusion processed fishmeal replacement diet comprising aquatic macrophyte greater duckweed (*Spirodela polyrhiza*) as D1, other as non-substituted fishmeal diet from pelettization D2. Diets are stored for six months (180 days) under four storage temperature conditions; -20°, 4°,ambient and 45°C namely T1,T2,T3 and T4, respectively. Changes in water-soluble and fat soluble vitamin profile is assessed bimonthly up to six month storage (at 0,60,120,180 days) for both the diets. Both diets at all storage temperatures, show substantial loss in water- miscible and fat-miscible vitamins namely A, E, K, B2, B12, C, 60-day further. Noteworthily in D2, intense diminutions in vitamin D content was observable during four month storage at T4 condition, while temperature, duration and interaction effects on vitamin D were significant ($P \leq 0.05$) for D1.Thiamin retentions in D2 are influenced by interference from antinutrients; while co-elutants affect B6 determinations in D2. Evidently, incurred loss of most vitamins were highest at end of storage under T4 conditions. The paper highlights effect of temperature, duration on dietary storage profile of vitamins as essential nutrients, affecting quality and shelf-life of stored processed feeds suggesting at best utilization regimes during storage.

Keywords: aquafeed, storage, temperature, vitamins, antinutrients, bioavailability.

1. Introduction

Fish is a valued source of quality dietary protein with reasonably significant extent of most indispensable amino acids, omega-n3 essential fatty acids - eicosapentaenoic, docosahexaenoic (EPA and DHA), minerals and vitamins. Digestibility and bioavailability of proteins, minerals and vitamins obtained from fish foods is higher compared to any plant-based food product. Fish is growingly becoming global staple; in fulfilling food requirement of world population rural as well as urban. Feed is the foremost important input for fish welfare and health in sustainable intensive aquaculture productions[1]. Demanding pursuit for alternative sources of protein and oil; wholly or partly substituting fish meal (FM) and fish oil (FO) in aquafeeds [2] accord to FAO recommendations on lessening their consumption from extract fishing, as well to meet sustainable UN goals for fisheries and aquaculture. In this direction much focus has shifted in finding and use of non-conventional alternative feed source towards aquaculture growth and developments [3,4]. To this effect potential of aquatic origin macrophytes such as duckweeds (belonging to family Lemnaceae) have been explored as alternative feed constituent. *Spirodela polyrhiza* and duckweeds at large offer abundant nutritional profiles significantly comparable to animal feed [5,6] with their partial inclusion in fish diets known to appreciably augment growth responses as well as nutritional attributes of fish [7].

Fish require vitamins in trace quantities as vital organic substances for important physiological functions. Vitamins are classified as water and lipid-miscible forms due to their miscibility in aqueous environment. Water-soluble vitamins comprise vit. B-group including in them B1, 2, 3, 5, 6, 7, 9,12 (namely thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folate, cobalamin) and ascorbate (vit. C). Vitamins (vit.) function mainly as coenzymes contributing to metabolic processes (B1, B2); in red blood cell formation, fatty acid- and amino-acid metabolism (B6, B12), amino acid interconversions (B6); bone formation, collagen synthesis and immunoregulatory functions (vit. C) [8]. Due to their hydrophilic property these vitamins are miscible in aqueous solutions. Vit. C is the most labile water-soluble vitamin, known for its antioxidant attributes. Vit. C is a co-substrate for enzymes involved in the biosynthesis carnitine, and neurotransmission. Fish like humans lack capability of vit. C biosynthesis and can fulfill it through dietary supplementation. Dietary inclusions of vit. C is found to improve growth, hematological parameters, carcass nutrients and feed conversion ratio in *Labeo rohita* [9]. Fat-miscible vit. A, D (calciferols), E (tocopherol), K are bound to lipophilic fraction in feeds and are transported, as well absorbed, in similar fashion as fats [10]. These vitamins have antioxidant properties (vit. A, E), hematological (vit. K) and immunostimulatory functions (A,D) hence contribute to fish and consumer health. Vit. A has essential function in vision, embryonic development, growth, reproduction, and differentiation [11]. Vit. E can prevent fragility of erythrocytes and due to its antioxidant mechanism prevent free radical oxidation of dietary (PUFA) and membrane lipids [12]. Loss of vitamins from diet is evident during processing, storage and due to leaching in aquatic conditions. Vit A, E and C due to their free radical sequestration potential have strong antioxidant properties. Thus, are prone to similar oxidative effects during storage. Storage loss of vit. A is reported due to oxidative influences [13]. Cessation of vitamins during prolonged storage (>180 days) is related to lipidic rancidity ensuing peroxidation [14]. Storage loss of vitamins require over compensations as supplementations to balance available levels from diet.

Storage feeds are prone to oxidative loss of lipids, proteins and vitamins as essential dietary nutrients. Autooxidation of lipids, proteins in feed can also decrease abundance of vitamins as natural antioxidants in feed ingredients [15]. Largely, diminution of feed vitamins is accentuated due to high temperature, humidity, extreme pH, light exposure, presence of elements and lipidic free radicals. Water soluble forms of vitamin are prone to storage effects showing losses due thermal, processing, storage and feed matrix properties. Extrusion processing of feed can affect nutritional quality of diet at various degrees. Diet composition along with extruder parameters including temperature, shear, screw type, pressure, and size of die have great influence on physical and nutritional quality of extruded feed [16]. Depending on extrusion conditions heat susceptible vitamins can be lost, with protein-sugar reactions at high extrusion temperatures deteriorating feed nutrient profiles [17,18] evaluated vitamin stability due to extrusion, and pelleting procedures at three months storage, reporting extrusion loss of thiamine (88%) compared to pelleting loss (60- 96%). Even coated forms of ascorbate are largely lost at high rates to extrusion. Most susceptible vitamins to extrusion were thiamine (due to high shear rates of extrusion), vit. A, C and folate. [19] evaluated impact of extrusion temperatures and encapsulation procedure on vitamin stability to conclude high stability of micro-encapsulated vitamin compared to unencapsulated forms in feed. Since many parameters need to be optimized for extrusion processing of feeds, the technique requires precise controlled operating conditions to restore maximal nutrient benefits from feeds on subsequent utilization.

Nutrient quality and safety are two most important attributes of healthy diet [4].There is a larger scope towards increase in fish production as well as their nutrient value for human consumption by judicious improvement in feed formulations and management of processed feed during storage, handlings; in farming environment. Storage help administer timely requirement of feed in aquaculture; nevertheless, improper storage or storage handlings can intensify feed spoilage [20]. Proper storage of feed is imperative for safeguard of feed quality, ensuring overall fish health and consumer beneficence. Knowledge of storage sequels help assess losses during prolonged feed utilizations, suggesting harmonization of stored processed feeds to maximize nutritional benefit for the fish and incidentally the consumers. The present study explores feed storage changes in composition of water-soluble, fat-soluble vitamins essential to fish nutrition in influencing overall feed quality during storage, at long duration variable temperature conditions. This work provides comparison of plant and fishmeal based formulated feeds to ascertain their response to the storage variables.

2. Materials and Methods

2.1 Formulation of feed

Aquafeeds were formulated using Interactive Fish Feed Designer (IFFD) software version 2 [21] as per composition given in table 1. Extrusion based compounded diet (D1), was formulated comprising fishmeal as major protein source with substitution of *S. polyrhiza* and wheat flour as minor protein sources, plant-oil sunflower oil was substituted for fish oil. Dry ingredients were well mixed with subsequent addition of oil and sifted through fine mesh, subjecting to extrusion pelleting with addition of extra pure milli-Q water (Synergy ultrapure, Millipore, Germany). Two-kilogram sinking pellet extruded feed was prepared based on twin-screw extrusion technique (BTPL lab model twin-screw food extruder, Kolkata, India) using 2.5 mm die as per set conditions with extruder rpm 214, cutter rpm 545, feeder rpm 10, extruder torque 13.9, heater1 (65°C) and 2 (70°C), temperature of final mass 101°C. Extruded feed was dried in feed drier (Hicon, India) for 4h at 35ºC, then kept at room conditions(1h) and packaged for storage in sealable bags. For D2 (non-extruded diet)hot water (60ºC) was added to well kneaded feed ingredients, subjecting to pelletization (Kent, India) through 2mm die. Prepared diet pellets were dried and packed for storage as D1.

TABLE 1: Diet composition

Diets	Ingredients	content (g kg^{-1})
D1, Extruded diet	wheat flour	276.45
	Spirodela polyrhiza	276.45
	fish meal powder	432
	sunflower oil (mL)	10
	vitamin-mineral premix#	4
D2, Non-extruded diet	wheat flour	471.69
	corn flour	75
	fish meal powder	528.3
	sunflower oil (mL)	10
	vitamin-mineral premix#	4

\# multivitamin tablet supradyn mg kg-1 of diet

2.2 Feed storage

Three replicates (n=3) per temperature condition were kept for storage. Diet samples at T1,T2 and T3 conditions were stored in resealable transparent plastic bags whereasT4 samples were kept in capped transparent glass bottles (Borosil, India) during storage. Daily temperature record was maintained towards T3 storage using thermo-hygrometer (ThermoPro TP53, US), table 2. Mean (± standard error, SE) temperature records for D1 ranged between 18.5ºC (±3.25) to 29.5ºC (±4.15) and D2 between 17.7ºC (±1.90) to 28.45ºC (±2.55). Replicates (n=3) for four temperature conditions under study were drawn bimonthly (at 0, 60th, 120th, 180th day) during each assessment.

TABLE 2: Ambient temperature record of stored diets

Storage Months	D1 Storage tenure (2021-2022)		D2 (2022-2023)	
	Maximum (º C)	Minimum (º C)	Maximum (º C)	Minimum (º C)
October	29	12	31	25.9
November	29	12	27	21.6
December	25	18.5	22	17.2
January	20	17	19.6	15.8
February	23.5	18	24.1	18.4
March	30.8	22.5	27.1	23.5
April	31.5	27.5	-	-

2.3 Preparation of feed sample

As per standard method 950.02, samples were milled to fine particles and sieved (1mm) to obtain fine powder [22]. Powdered diet was thoroughly mixed before analysis of vitamin profile. Each assay was performed in replicates for calculation of mean for the evaluation of results.

2.4 Vitamin analysis

2.4.1 Chemicals and Reagents

Vitamin standards, retinyl acetate (vit. A) (No.46958, Sigma-Aldrich Chemicals private limited, India), thiamine hydrochloride (B1), riboflavin (B2), pyridoxine hydrochloride (B6), cyanocobalamin (vit. B12) and L-Ascorbic acid (vit. C) (No. 47858, 47861, 47862, 47863, 47869 respectively), cholecalciferol (vit.D3), ergocalciferol (vit. D2), vit. K1 (No. 95271 Bio-xtra sum of isomers, HPLC grade) at high purity >98% were purchased from Sigma-Aldrich, USA. E-acetate was obtained from Himedia laboratories private limited, India at purity >97%. Taka-diastase, source *Aspergillus oryzae* (No. 86247) was obtained from Sigma-Aldrich (USA). n-butanol, chloroform (SRL, Sisco Research Laboratories, India), acetone, sodium acetate (extrapure AR SRL, Sisco Research Laboratories, India), sulphuric acid (Merck, India), methanol, acetonitrile (HPLC grade, Merck, India), metaphosphoric acid (No.239275, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were also procured. Standard solutions were prepared using extrapure Milli-Q water at pH7.

2.4.2 Sample preparation for vitamins

Water-soluble vit. B1, B2, B6, B12 and C, were assayed according to the method of [23]. Extraction was performed in triplicates. For vit. B1, B2, B6 and B12 estimation diet sample (2g) was added with 25mL H_2SO_4 (0.1N) solution in a conical flask. Sample solution was incubated for 30min at 121°C in oven. Contents were cooled at room temperature and using 2.5M sodium acetate, pH was adjusted to 4.5. Then, 50mg Taka-diastase enzyme powder was added to the sample. After gentle vortex for 1min, preparation was stored in oven at 35°C for 18h. The digested mixture was filtered through Whatman filter (No.4). Filtrate was diluted with 50mL Milli-Q ultrapure water. Extract was re-filtered through 0.45μm micropore syringe filter, into HPLC vial. For the estimation of vit. C, 10g diet sample was blended and homogenized with 25mL extracting solution comprising 0.3M metaphosphoric acid and 1.4M acetic acid. After 15min centrifugation at 10,640g (fixed-angle rotor 12181 Sigma 2-16K, Germany)mixture was filtered using Whatman filter (No. 4). Fat-soluble vitamins (A, D, E and K) were estimated following the method of [24]. Briefly, in a falcon tube 0.5g sample was mixed with 8mL chloroform and acetone mixture in the ratio 70:30. Nitrogen (N2) gas was flushed through the sample and tube was tightly capped. Contents were vortexed for 1min and tube was left to stand for 5min then vortexed again for 30s. Mixture was centrifuged at 1702g (fixed-angle rotor 12181 Sigma 2-16K, Germany), 25°C for 5min. After centrifugation, 1mL supernatant was transferred for drying in a N_2 evaporator. Completely dried extract was dissolved in 1mL n-butanol. Reconstituted extract was filtered using 0.4 μm syringe filter and kept in HPLC vial for further analysis.

2.4.3 Determination and quantification

Stock solutions of vitamin standards 1.0mg mL^{-1} were prepared. Standard calibration was performed to obtain linearity at concentrations 0.25, 0.5 and 1.0mg mL^{-1}. Chromatographic analysis of vitamins was conducted by ultra-high pressure liquid chromatography (UHPLC Thermo Fisher, model Dionex Ultimate 3000) with stationary phase C18 column (3μm,150x4.6mm). 20μL of sample extract was injected into the HPLC system. Simultaneous separation for vit. B1, B2, B6, B12 was achieved at a flow rate of 0.5 mL min^{-1}, wavelength 270 nm using MeOH : 0.023M H_3PO_4 (33:67 ratio, pH 3.54) as mobile phase. For estimation of vit. C, flow rate of 1mL min^{-1}, wavelength 254nm and acetonitrile (ACN, 100%) as mobile phase solvent was deployed. Fat-soluble vit. A,D,E,K were determined at flow rate 1.8 mLmin^{-1}, UV absorbance 290nm, 100% MeOH as mobile phase.

2.5 Statistical analysis

For both fat and water-soluble vitamins, data is represented as mean SE. Analysis of variance (two-way ANOVA) considering major effects of temperature, storage duration and interaction on vitamin content of formulated feeds is performed using SPSS statistics (IBM, version 25)[25]. Statistical differences are reported by honestly significant difference (HSD). Level of significance $P \leq 0.05$based on Tukey's post-hoc analysis is considered[26].

3. Results and Discussions

3.1Water-soluble vitamin storage changes

3.1.1 Vitamin C

Among all storage temperatures, vit. C retention at end of 180 day storage duration is highest at T1 condition, (Fig.1a,1b), in order T1 (89% D1, 86.13% D2) > T2 (85.3% D1, 81.02% D2) >T3 (51.38%

D1, 79.56% D2) >T4 (39.45% D1, 51.82% D2) retentions. Significant difference ($P \leq 0.05$) due to temperature, duration and interaction effects was found for vit. C values in D1. For D2, storage effects of duration were highly significant. Neither temperature nor interaction had significant effect on changes in vit. C for D2. Although D1 and D2 depict gradual loss of vit. C over low temperature storages (T1,T2 and T3); losses are evidently higher at T4 due to intense temperature effects. This is in agreement with works of [27, 28], reporting decreased vit. C levels in formulated feeds due to extrusion, pelletization processing based moisture and heat addition, causing rapid losses at extreme conditions. Vit. C in aquafeeds is susceptible to processing, storage losses [29]. Additionally, oxidative, non-oxidative and leaching losses of vit. C in fish feeds are reported. In fishes, dietary deficiency of vit. C can impair collagen synthesis leading to scurvy, lordosis, scoliosis, impaired wound healing, lethargy and anemia symptoms [29, 30]. [31], recommends 67.17 mg kg^{-1} of ascorbate in grass carp feed for optimum growth parameters of juveniles. Reducing potential of vit. C can be utilized by its supplementation to diets containing high levels of polyunsaturated fats thereby, preventing oxidation of feed lipids [32]. Vit. C potentiates antioxidant effects of vit. E in protecting lipids against peroxidative distress [33].

VITAMIN C

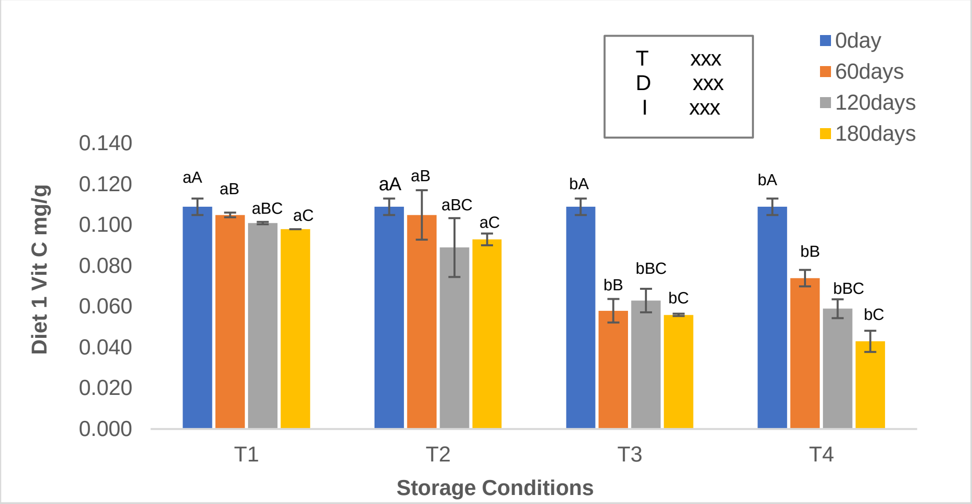

Fig.1a

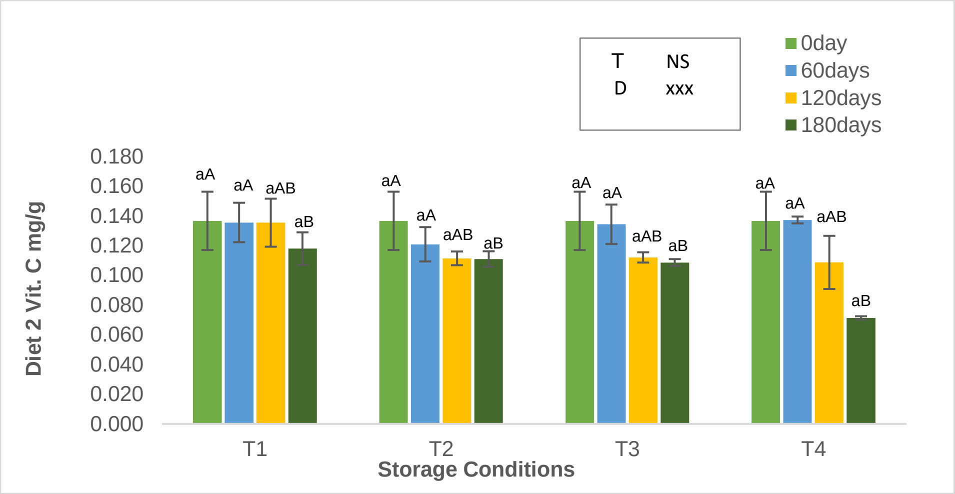

Fig.1b

Fig.1a-1j Storage changes in water-soluble vitamins in D1 and D2

[#] values are mean ± SE (n=3), statistical differences (Tukey's, $P \leq 0.05$) in means as changed letters: changed small letters 'a - c' in similar column denote difference is significant ($P \leq 0.05$) among temperature conditions; changed capital letters 'a-d' in similar row denote difference is significant ($P \leq 0.05$) among feed storage durations. p-value for between subject effects of temperature (T), duration (D), interaction of temperature x duration (I) shown as xxx ($P \leq 0.001$), xx ($P \leq 0.01$), x ($P \leq 0.05$) for significant P-values; NS (not significant); ND (not defined).

3.1.2 Vitamin B1 (thiamin)

Gradual decrease in vit. B1 is evident bi-monthly at all storage temperatures for D1, (fig.1c). Incurred losses being higher at high temperatures T4 (55.81% vit. B1 present) >T3 (61.88% present) compared to higher retentions at low temperatures T1 (66.06%), T2 (66.17%) at end of storage duration. Thiamine is thermolabile and susceptible to losses at high temperature processing (extrusion) as well as low moisture conditions. Extrusion loss of thiamin up to 50% is reported by [18]. As per [8] thiamin retentions ranged between 60-80% in extruded feeds stored for 3months at ambient temperatures. Although, non-extruded D2, show initial increase in thiamin concentration up to 120 days, with decreases at later duration between fourth to sixth month across storage temperatures, (fig.1d). These findings are in good agreement to study based on frozen foods [34], reporting initial increase in thiamin content during storage, processing; followed by storage decrease of thiamin, yet no proposed mechanism could be explained. We believe that this effect may be due to interaction effects of thiamin with dietary antinutrients mainly phytochemicals such as tannins, which would have overestimated vit. B1 peaks in chromatographic run. Additional work by [35] provide support to this hypothesis on thiamin-tannin interaction indicating formation of adducts (weak bonds) between thiamin and oxidized tannin to be coeluted at same retention values during thin-layer chromatographic analysis. Compared to extruded diets, non-extruded diets are prone to interaction influence of polyphenols. Extrusion processing can reduce tannins and their interference on bioavailability of dietary nutrients (proteins, lipids, vitamins and minerals). Duration effects on storage thiamin are significant for both diets while temperature and interaction significantly ($P \leq 0.05$) impact thiamin concentration only in D2.

VITAMIN B1

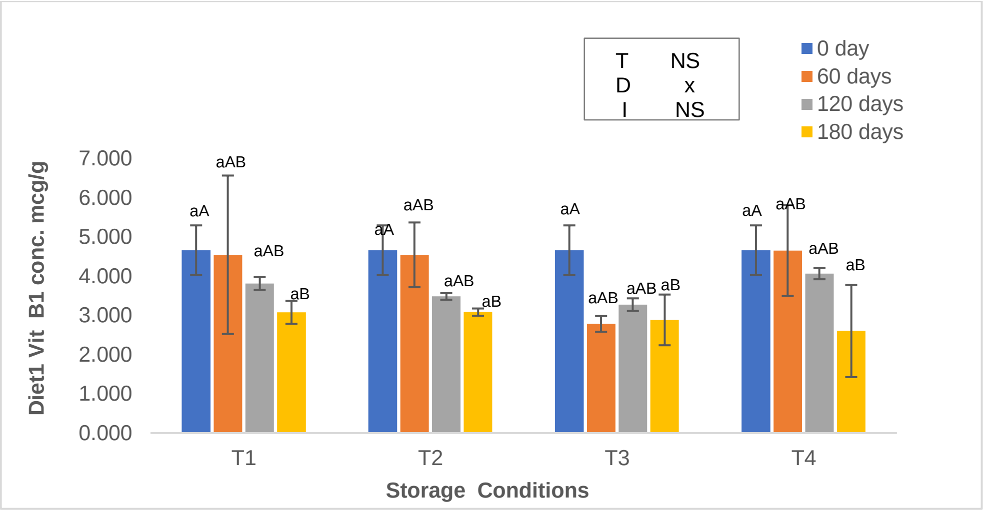

Fig.1c

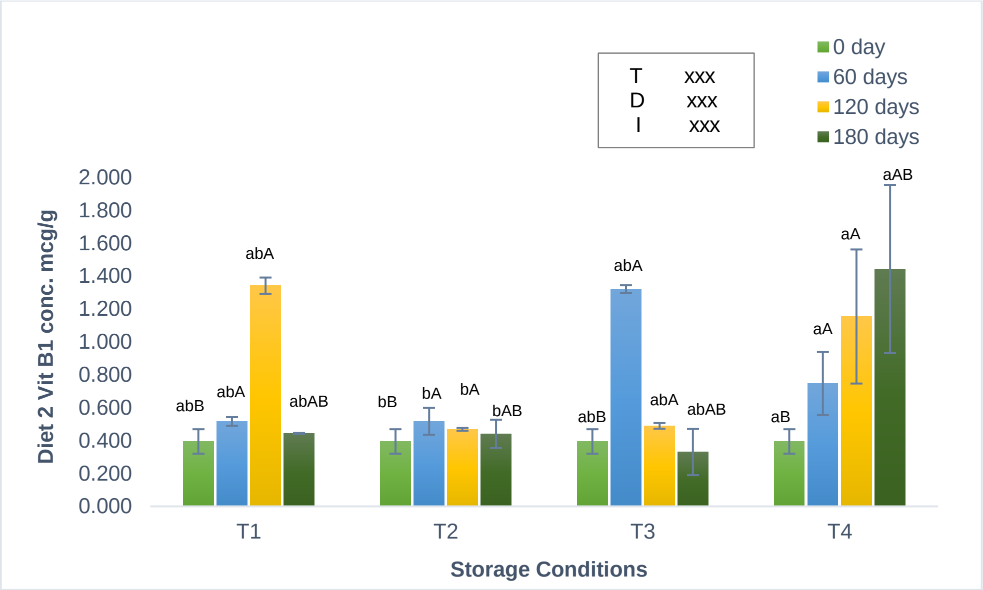

Fig.1d

3.1.3 Vitamin B2 (Riboflavin)

Vit. B2 in D1 show significant (P≤0.05) duration effects with decrease from initial to two-month storage at all temperatures. Effects of temperature and interaction on B2 changes are insignificant for D1; whereas temperature, duration, and not interaction impacts vit. B2 concentrations in D2 significantly (P≤0.05). [36] described higher heat loss of riboflavin compared to thiamin. Increasing temperatures relate to decreases in riboflavin retention [37, 38]. It is noteworthy that vit. B2 decline intensely over storage durations in D1 compared to D2, (fig.1e,1f). In D1 at 60th day, vit. B2 value at T1 decreased from initial concentration of 0.352 to 0.003 mcg g^{-1} (with loss of 99.15% that is merely 0.85% retention) and remained undetected beyond two months. At T2, 60-day conc. depicts 15.34% retention (0.054 mcg g^{-1}), at T3 6.57% is retained (0.023 mcg g^{-1}), T4 (7.95% retention at 0.028 mcg g-1). Vit. B2 could not be determined in D1, at any storage temperature during fourth month and at T1, T4 during sixth month. Extrusion loss of riboflavin is large up to 50%. These losses due to extrusion are dependent on increasing shear rates due to increase in screw speed, moisture [19]. Extrusion does not have direct thermal effect on B2 recoveries, rather it increases B2 loss by reducing residence time achieved by increasing screw speed and moisture decrease [18,39]. For non-extruded D2, vit. B2 show significant loss at each storage interval compared to initial concentration. Retention values of vit. B2 at second, fourth and sixth months were determined respectively at T1 as 96.49%, 84.21%, 57.89%; T2 as 96.49%, 78.95%, 71.93%; T3 as 66.67%, 38.60%, 42.11%; and, T4 as 29.82%, 0%, N.D. (not defined at end of storage). This is in agreement to [19,39]; elaborating higher retention rates of riboflavin under optimal conditions of processing. Being thermostable, B2 is less prone to temperature effects compared to thiamin; unless affected by processing parameters, composition of food matrix, oxidation, pH, moisture and presence of oxygen. Riboflavin is a light sensitive vitamin. Its stability is affected by oxygen, moisture and water activity [40]. Presence of oxygen during storage can severely accelerate B2 deterioration rate [41]. Photosensitization (visible or UV light exposure) of riboflavin, under oxygenated conditions can form reactive singlet oxygen further capable of oxidative degradation of other vitamins - A,C,D and E along with dietary proteins and lipids [40]. Packaging material plays an important role in B2 photostability. Intense losses of B2 can occur due to improper exposure to light from package material even when other conditions of storage (pH, temperature, moisture) are optimum [42]. Rapid loss of riboflavin from milk packaged in transparent bottle, sachet has been reported, compared to light protected milk packaging in carton or dark bottle [43]. Since transparent packaging was used for storage packaging, following may be the reason for vit. B2 deterioration in our storage diets.

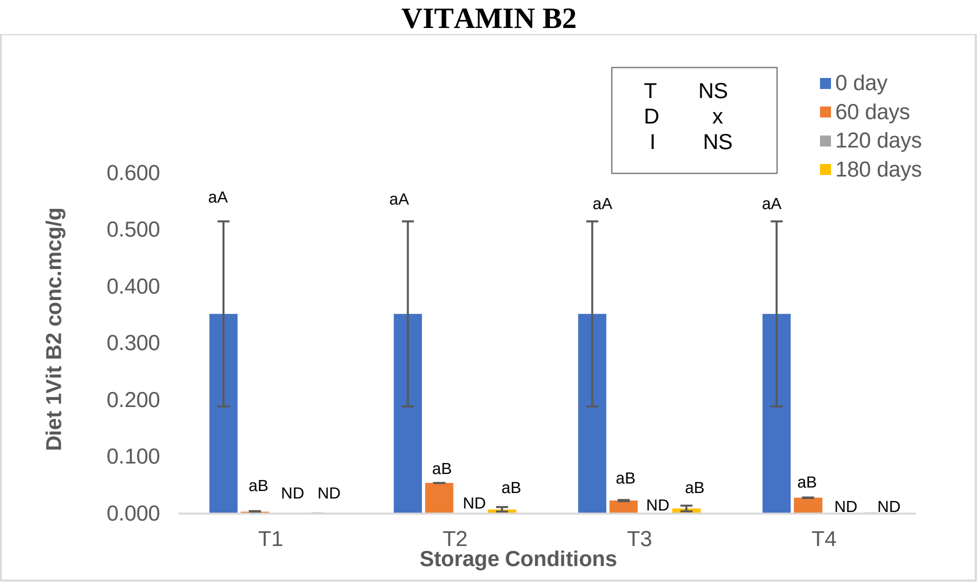

Fig.1e

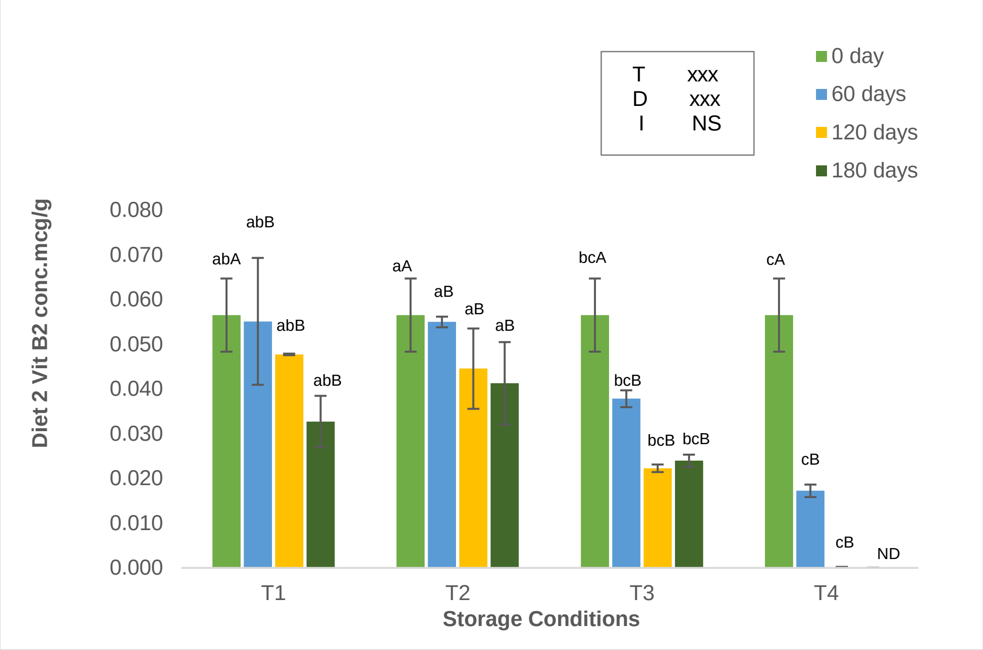

Fig.1f

3.1.4 Vitamin B6 (pyridoxine)

In D1, vit. B6 retention at end of 60,120,180 day followed 69.48%, 61.49%, 56.76% respectively at T1; 88.63%, 72.52%, 83.44% at T2; 98.53%, 75.90%, 74.54% at T3; 90.20%, 73.19%, 46.51% at T4, (fig.1g). D1 results show significant effects ($P \leq 0.05$) of temperature, duration and interaction. D2 depicts significant decrease in retention of B6 at end of storage at all temperatures, with T1,T2,T3 values at 80.37%, 80.74%, 71.48%, except with slight chromatographic overestimation at T4. Overestimations compared to initial storage content are also noteworthy for D2 at 60th day at T2, T3,T4 storage and 120th day T1, T3,T4 conditions (fig.1h). This trend may be due to artifacts of enzymatic activities (here taka-diastase) on release of bound forms vit. B1, B6 and B12 [44] as well as chromatographic overestimation due to co-elutions; a problem in simultaneous estimation of vitamins during single chromatographic run [45]. Vit. B6 exists as six distinct forms of which three vitamers- pyridoxal, pyridoxine and pyridoxamine (PL, PN, PM respectively) are relevant in chromatographic determinations.

VITAMIN B6

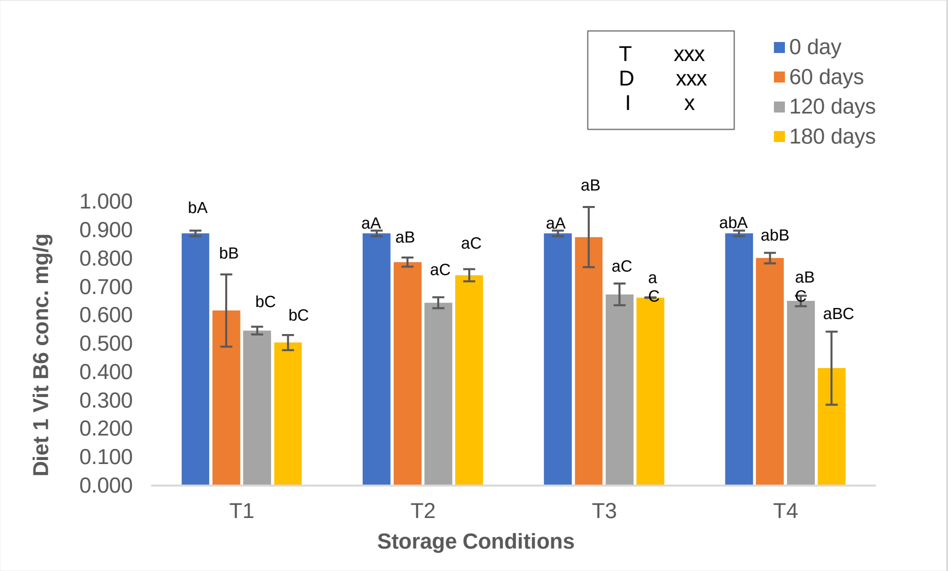

Fig.1g

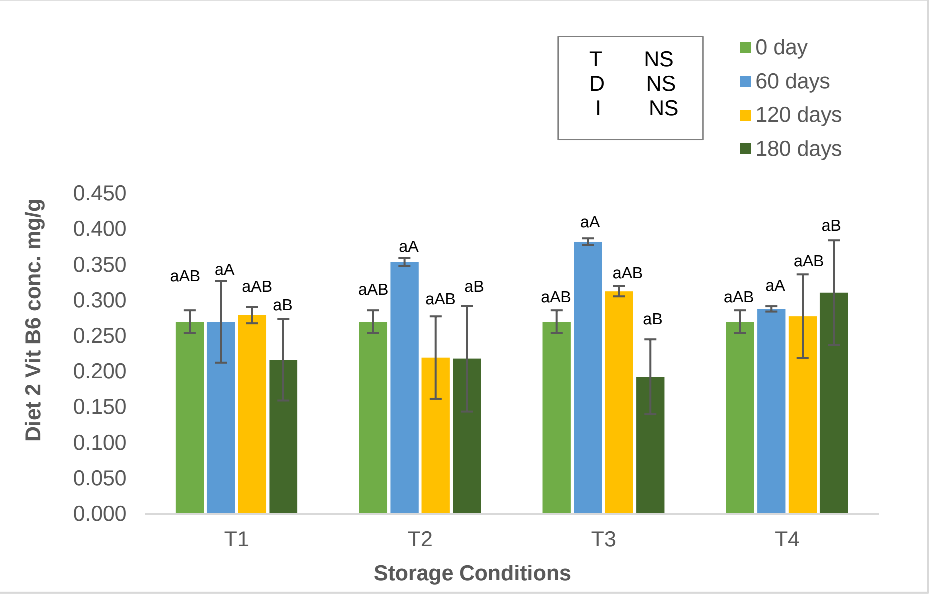

Fig.1h

Presence of several bioactive forms of the vitamins, along with their physicochemical heterogeneity make simultaneous analysis difficult task. Variable forms and properties of B6 vitamers complicate analysis of pyridoxine [46]. Thus, applications reporting food estimations determine B6 concentration as sum of vitamers PL+PM+PN [47], PL and PM being interconvertible forms [48]. In plant-based foods PN dominates; while PM, PL are found in animal origin foods. All these forms (PL, PM, PN) exist in compounded diets consisting of plant and animal-based ingredients. According to [44], enzyme protocol in vit. B6 estimation increased their content due to the release of bound forms from food matrix; this release being under effect of enzyme concentration, purity and differential ability of enzyme combinations used to release specific forms of B6 vitamers.

3.1.5 Vitamin B12 (cobalamin)

In both diets, at each temperature condition variations in vit. B12 content exhibited duration based significant effects ($P \leq 0.05$), between initial and final storage. Temperature and interaction effects are not visible in vit. B12 changes in D1(fig.1i).

VITAMIN B12

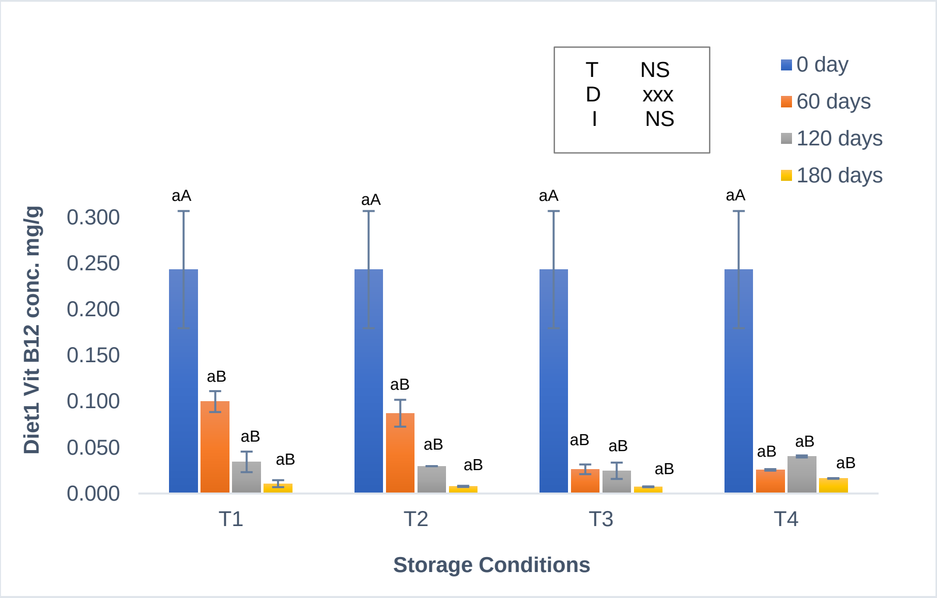

Fig.1i

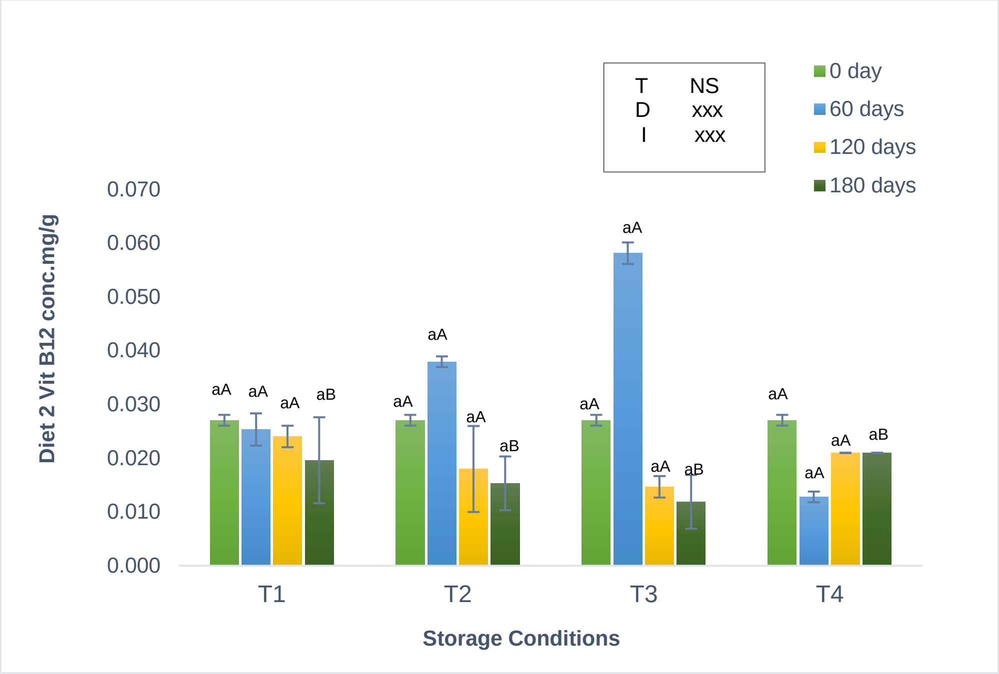

Fig.1j

For D2, duration and interaction effects are highly significant for changes in vit. B12 value, but no noteworthy effect of temperature lies for both diets during storage (fig.1j). T1 retention of cobalamin estimated at 45.27% for D1, 74.07% (D2); T2 (32.92% D1), (55.56% D2); T3 (28.81% D1), (49.38% D2); T4 (65.84% D1), (77.78% D2) clearly with higher vit. B12 retentions in D2 during storage. Although T1, T2 retentions for vit. B12 are higher compared to T3, in both diets at the end of storage; T4 values at 180-day in both diets are highest although this increase at T4 is unexplainable. As per [39] few studies are available on cobalamin; and possibly fewer about its storage behavior in fish feeds limiting our knowledge on the possible effects of variables on cobalamin changes. Present work may fulfill this gap in literature.

3.2 Fat-soluble vitamin storage changes

Fat-soluble vitamins are also prone to effects of storage and feed processing variables. Moreover, lipid oxidation in stored diets yield hydroperoxides that reduce availability of fat-soluble vitamins in storage feeds [20].

3.2.1 Vitamin A (retinyl acetate)

At each bimonthly assessment vit. A decreased at all storages temperature in both diets. In D1 end of duration vit. A retentions follow T1(72.52%), >T2 (38.65%), >T3 (20.96%), >T4 (3.26%) showing decline across increments in storage temperatures (fig.2a). Similar trends are observable towards vit. A retention rates in D2, at 71.37% (T1), 68.46% (T2), 24.48% (T3), 4.98% (T4), fig.2b. Under feed storage regime higher vit. A retentions in D2 than D1 explains less severe effects of pelleting compared to extrusion. Temperature affects vit. A values in D2significantly ($P \leq 0.05$), between low temperature (T1,T2) and other temperature conditions (T3,T4), between T3 and T4. Between subject effects of temperature and duration on vit. A storage depletion are highly significant (P=0.000) for both diets, with significant interaction effects only towards D2. High temperatures above 100°C, moisture, extrusion conditions (such as screw speed) have extreme impact on vit. A depletion [13,18,49].Vit. A has essential function in vision, embryonic development, growth, reproduction, and differentiation [11]. Vit. A is extremely labile to oxidative loss arising from lipid, protein oxidations following free radical attacks.

VITAMIN A

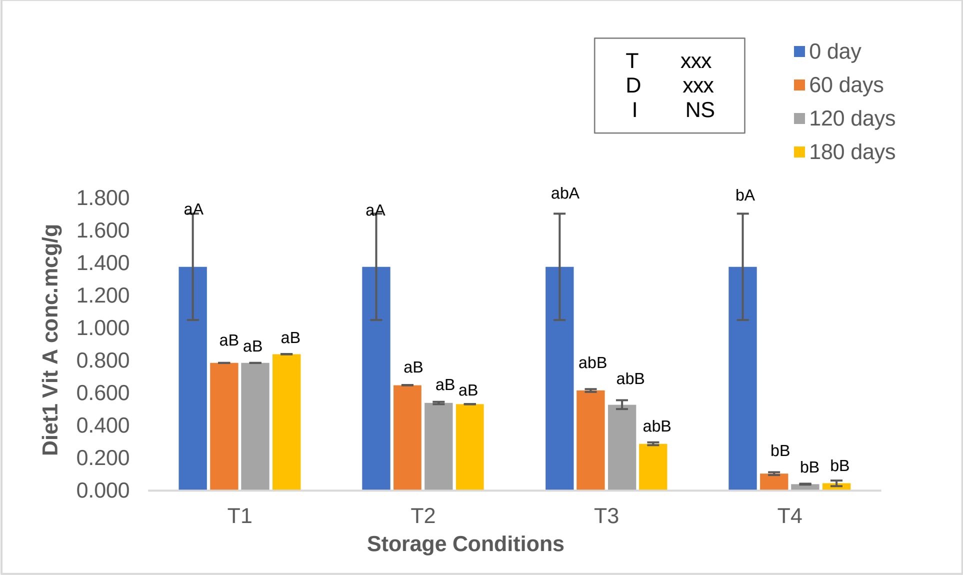

Fig.2a

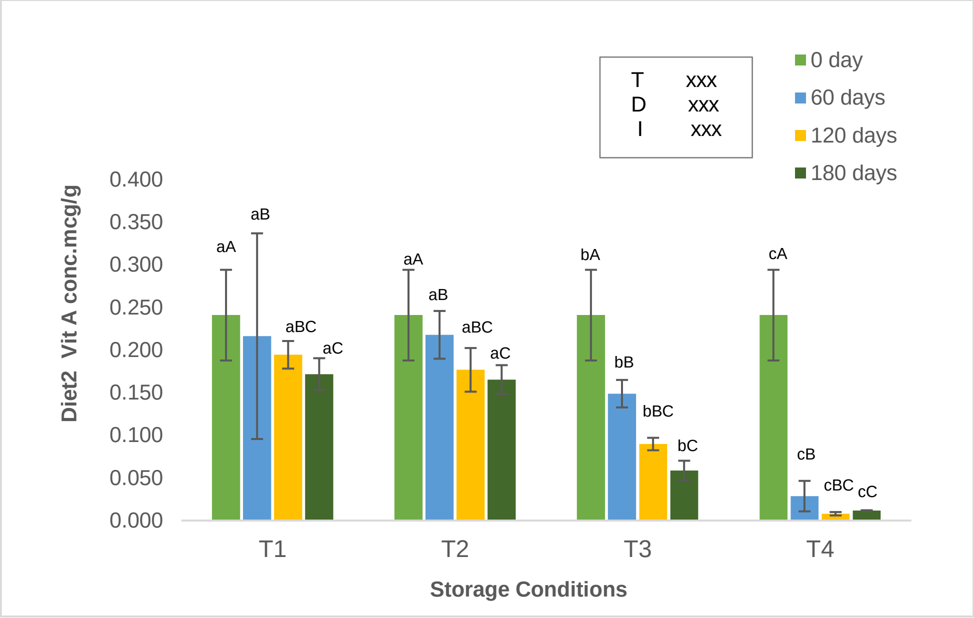

Fig.2b

Fig.2a-2i Storage changes in fat-soluble vitamins in D1 and D2

values are mean ± SE (n=3), statistical differences (Tukey's, P≤ 0.05) in means as changed letters: changed small letters 'a - c' in similar column denote difference is significant (P≤ 0.05) among temperature conditions; changed capital letters 'a-d' in similar row denote difference is significant (P≤ 0.05) among feed storage durations. P-value for between subject effects of temperature (T), duration (D), interaction of temperature X duration (I) shown as xxx (P≤ 0.001), xx (P≤0.01), x (P≤ 0.05) for significant P-values; NS (not significant); ND (not defined).

3.2.2 Vitamin D (vit. D2 ergocalciferol, vit. D3 cholecalciferol)

Detectable vit. D in D1 is plant-based form ergocalciferol (vit. D2). For D2 animal-based form cholecalciferol (vit. D3) was quantifiable. Estimated retention of vit. D2 (in diet D1) at end of storage accounted 99.40% (T1), 50.57% (T2), 30.21% (T3), 39.54% (T4), fig. 2c. Severe losses of vit. D3 were encountered in pelleted stored diet with incurred losses accounting 100%, fourth storage month onwards at T4 storage. Even at T1,T2 and T3 accountable vit. D3 retentions were low as 38.46%, 26.92%, 7.69% respectively (fig.2d). These results are in alignment to [36],depicting high pelleting losses of vit. D3 nearing 80-90% with stable extrusion changes. Only duration effects (P=0.000) are noteworthy for vit. D3 changes over storage, with no significant effect due to temperature (P=0.084) or interaction (P=0.442). Alterations in vit. D2 was significantly (P≤0.05) under impact of temperature, duration and their interactions as well.

VITAMIN D

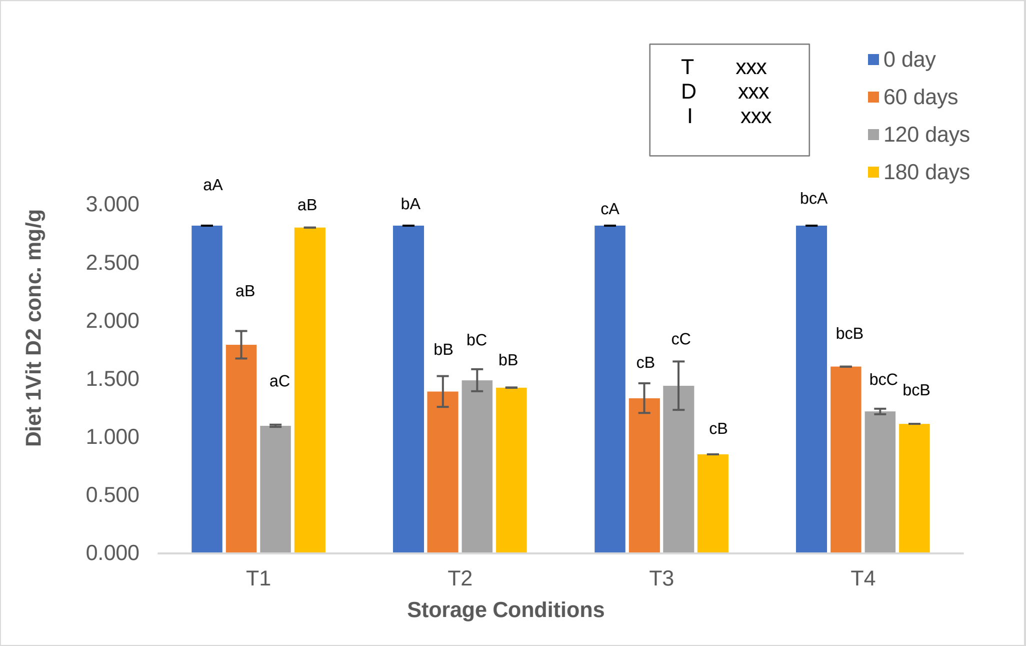

Fig.2c

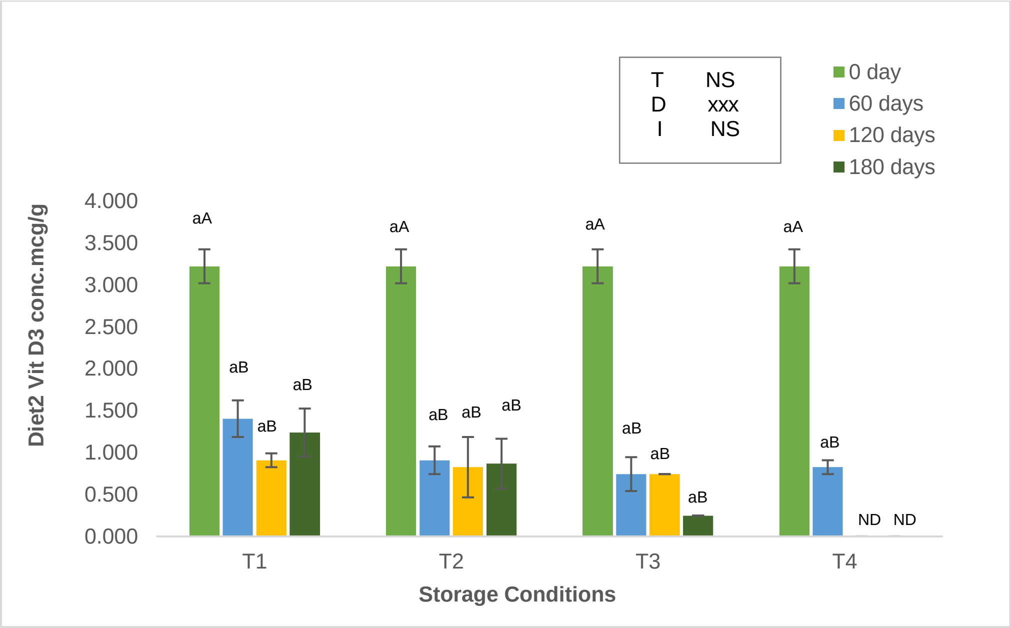

Fig.2d

3.2.3 Vitamin E (tocopherol)

Vit. E loss ranging from 39.41% to 51.36% are estimated for extruded diet D1, at end of storage, (fig.2e). At storage culmination, pelleted D2 loss accounted between 59.78% - 80.43% (fig.2f).Variabilities in dietary loss of tocopherols may be with effect to thermal oxidation, degree of lipid rancidity, presence of light, or metal chelators typically zinc, iron, and copper [50]. Vit. E can prevent fragility of erythrocytes and due to its anti-oxidant mechanism prevent free radical oxidation of dietary (PUFA) and membrane lipids [12]. Dietary vit. E requirements for rohu fry is determined to be 131.91 mgkg-1 dry weight of feed by [12] and diet conc. 120 mgkg^{-1} for Atlantic salmon [51]. Vit. E along with vit. A, C have strong antioxidant properties owing to their free radical sequestration potential. Thus, are prone alike to oxidative effects during storage.

VITAMIN E

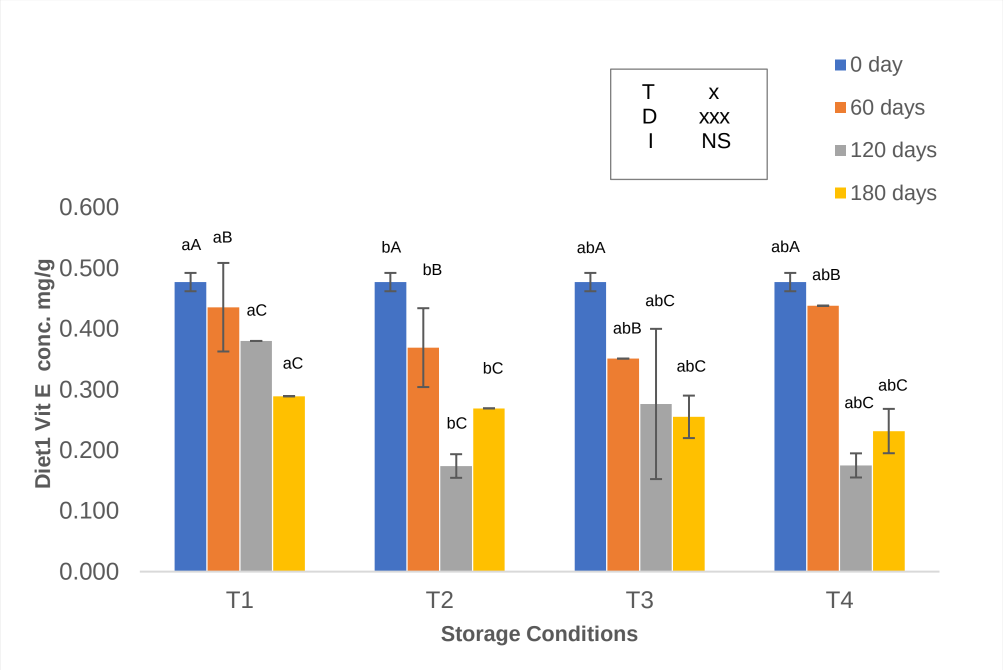

Fig.2e

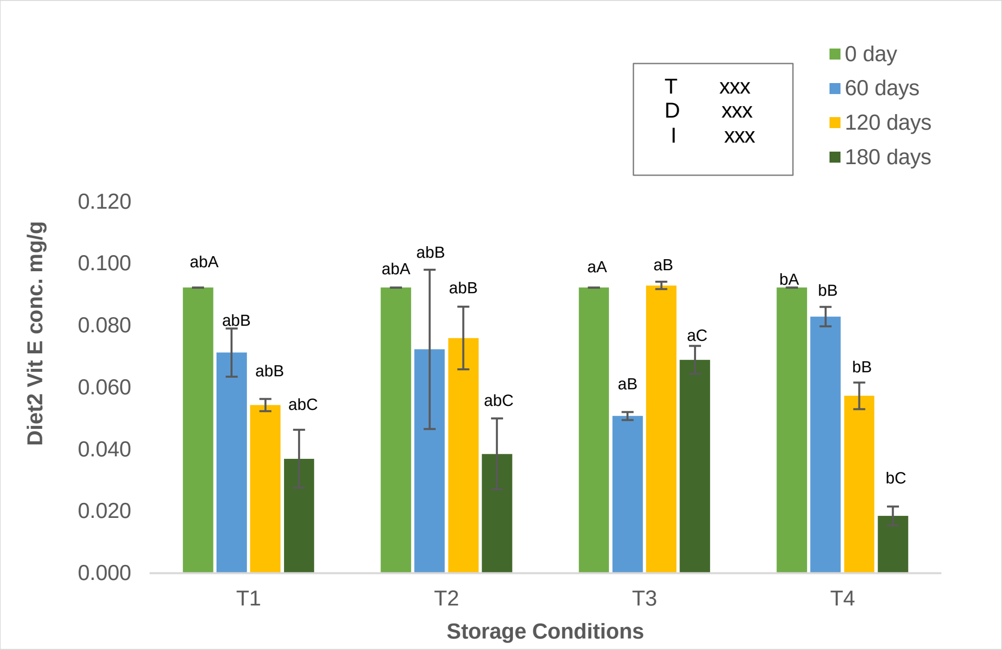

Fig.2f

3.2.4 Vitamin K1 (Phylloquinone)

Vit. K1 in feeds was identified as trans- and cis-isomers (fig.2g-2i) at chromatographic retention times (RTs) 1.09-1.15 for cis-, 1.77-1.807 for trans- isomer. Of these, only trans-form of vitamin K1 is biologically relevant [52]. K1-trans, during storage, in both diets (fig.2g, 2h) show significant between subject-effect for duration; but not for storage temperature and interaction. Incurred losses are higher for extruded diet (between 41.28% to 64.41%), than pelleted D2 (4.81%-49.89%).

VITAMIN K

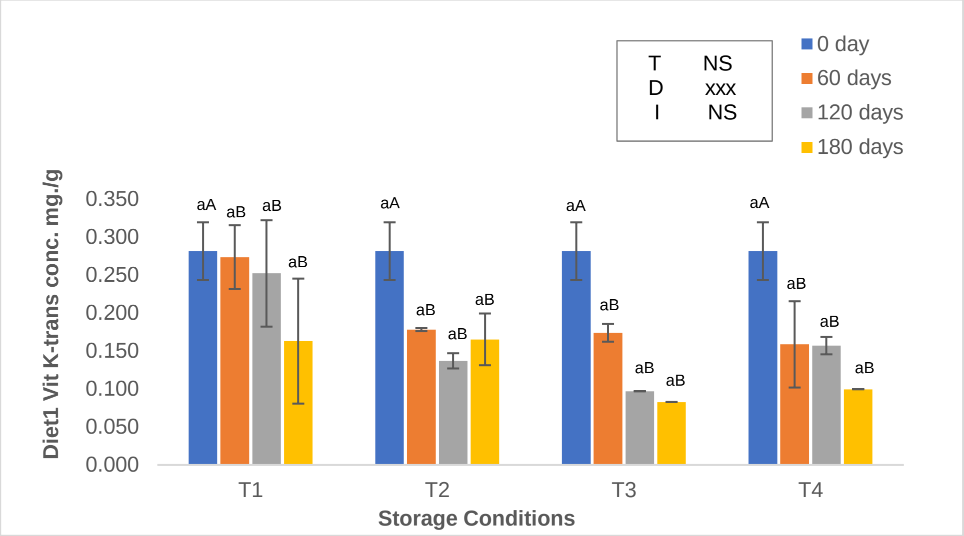

Fig.2g

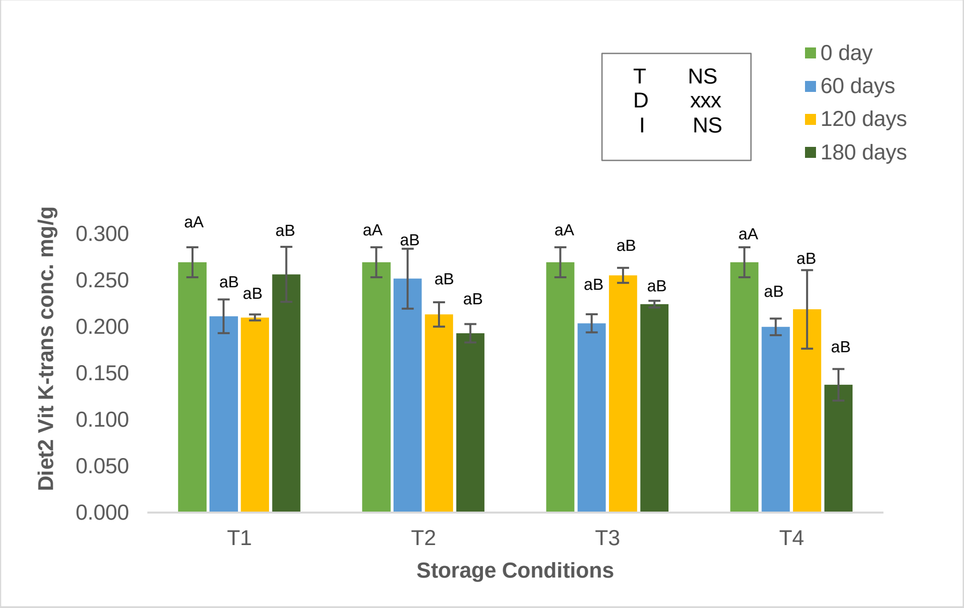

Fig.2h

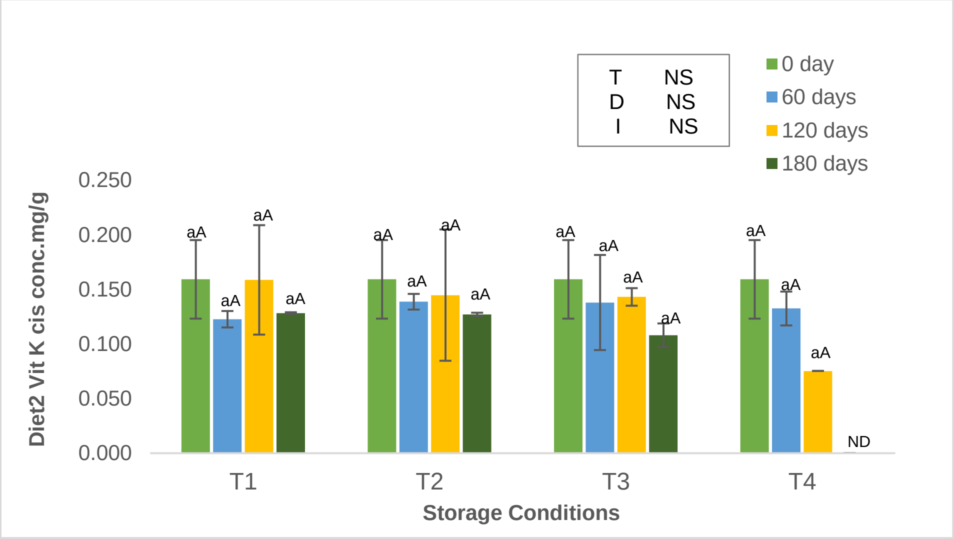

Fig.2i

4. Conclusions

The study evaluates effect of storage duration, temperature and their interaction, on vitamin profile in fish feeds prepared from extrusion and non-extrusion pelletization procedures. For both diets, substantial storage loss in water- miscible (B2, B12 and C), and fat-miscible (A, E, K) vitamins is evident from 60day onwards, at all storage temperatures. Intense diminutions in vit. D content was observable during four month storage at T4 condition for D2; while storage temperature, duration and interaction effects on dietary vit. D were significant ($P \leq 0.05$) for D1. D2 retentions for thiamin are influenced by interference from antinutrient factors in feed; while co-elutants affect determination of vit. B6 in D2. Through the study, it is evident that depletion in essential micronutrients with regard to feed vitamins incurs severe loss to feed quality. Incurred loss of most vitamins were highest at end of storage under high temperature T4 conditions. These diminutions are caused by oxidative as well as non-oxidative changes in vitamins along with interactive consequences of other dietary nutrients (proteins, lipids, elements/metals)on vitamin profile during storage. It is noteworthy that vitamin analysis and estimation is strongly influenced by processing technique, co-elution and antinutrient interferences during chromatographic run. Furthermore, the study helps cognize impact of temperature and duration on feed quality at storage inventories, and in farming environments. Storage depletion of vitamins signify need to develop feed formulae taking into consideration the percentage loss of each nutrient from the foodstuffs, specifying at strategic formulation of feed keeping in account storage impacts. Additionally, these findings offer helpful information for fish farmers in managing storage of formulated feeds, with an aim to prolong their shelf-life safeguarding significant amount of the total production costs of fed aquaculture and incidental consumer health.

Acknowledgements

Authors are thankful to their institutions Maitreyi College, University of Delhi and Delhi Technological University for constant support towards formalization of the research.

Author contributions

Parul Puri: experimentation, literature search, conceptualization, writing-original draft preparation, analyzed the study, formal analysis. **Neha Tiwari:** standardization and performance of vitamin determination, formal analysis. **Vaibhav Puri:** Formal analysis, result interpretation; **Ram Singh, Jai Gopal Sharma, Rina Chakrabarti:** supervision, writing-reviewed the overall manuscript, edited, critically revised drafted work. All authors agreed on the final draft.

Statements and Declarations

Competing Interests: Authors declare that there are no financial or non-financial interests directly or indirectly related to the work submitted for publication also there is no conflict of interest in regard to same.

Funding: This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data Availability: Data available from corresponding author on reasonable request.

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