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Alternative proteins support somatic and muscular development while remodeling the microbiome in zebrafish

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Protein intake is fundamental to growth, well-being and long-term health. Unfortunately, many diets rely upon animal-based proteins, which are environmentally costly. To feed a growing population, alternative protein sources will be necessary. To determine the health implications of switching entirely away from animal-based diets, we fed alternative proteins to a model vertebrate during development. Zebrafish were fed diets including protein from fishmeal, pea, milk and whey, and their growth and health were monitored. Most diets supported growth, with the exception of those high in whey and milk protein, which resulted in fish that were ~10% shorter in body length and had muscle fibers ~30% smaller than control. Of interest, genes associated with insulin sensitivity and fat storage were upregulated in some diets (*lepr*, 2 to 3.5 fold, and *fasn*, 2.5 to 4 fold, respectively). The microbiome changed dramatically between animal and alternative proteins, shifting from Fusobacteriota to Proteobacteria dominance, with *Cetobacterium* positively affecting health, and *Aeromonas* doing the opposite. Our findings indicate that more environmentally friendly diets can lead to healthy outcomes, but that the protein source is critically important.

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Introduction

A nutritious diet balanced in macro- and micronutrients is the cornerstone of growth, development, physiological well-being, and long-term health.[1,2] Diet not only contributes to one's lifespan, but also one's health span—or overall quality of life.[1] Both early and late-life nutrition can profoundly influence processes such as musculoskeletal growth, cardiovascular health, energy metabolism, neurodevelopment, the gut microbiome, and immune function.[3–5] Our food choices are clearly critical to our own health, but are also critical to the health of the environment that supports food production.

In recent decades, what we eat—and how that food is produced—has increasingly become a cause for concern due to potential adverse effects on human health and the environment.[6] It is now understood that poor-quality diets are the foremost risk factor for the pathogenesis of non-communicable diseases, including obesity,[7,8] cardiovascular disease,[7,8] diabetes,[7,8] and cancer[9]—irrespective of age, sex, or demographic.[9] Poor-quality diets and malnutrition account for nine of the top 15 global morbidity risk factors,[10] and are responsible for approximately 11 million preventable deaths annually.[9] Dietary choices also impart downstream effects that extend beyond the individual, impacting environmental and planetary health.[11]

Ongoing advancements in food science aim to develop sustainable, yet nutritious, dietary options.[6,12] At the forefront of this shift are alternative proteins—plant-based and food technology alternatives intended to replace or supplement conventional animal-based protein options.[13,14] Recent meta-analyses indicate that replacing meat consumption with high-quality plant proteins can improve blood lipid profiles,[15,16] enhance glycemic control,[16–18] and reduce the risk of developing obesity,[16,19] cardiovascular disease,[15,16,18,19] diabetes,[16,20] cancer,[16,19] and total mortality.[15,18,19,21] Second, alternative proteins have the potential to diminish our reliance on animal-derived foods with large environmental burdens—such as ruminant meat and dairy—which account for approximately 80% of food-related greenhouse gas emissions.[22,23] Finally, alternative proteins offer a scalable solution to meet growing protein demands while ensuring food production remains within the confines of planetary carrying capacity.[12] However, protein sources differ in their amino acid composition, ratios, and bioavailability,[2,24] which raises dietary concerns for transitioning to alternative protein sources.

Despite these promising benefits, current research on alternative proteins has several notable shortcomings. Most

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studies have focused on short-term dietary interventions in adult humans or rodent models, often overlooking critical developmental stages such as adolescence, when nutritional requirements are high and long-term trajectories are established.[25] This is particularly important, given evidence that adolescent interventions can have more profound and lasting effects compared to those in adulthood.[26,27] The potential metabolic, developmental, and physiological impacts of substituting animal protein with alternative sources across the entire growth period remain unclear, particularly in controlled experimental models. This study addresses these gaps by using zebrafish as a vertebrate model to examine the long-term effects of substituting animal protein with alternative sources from early life (30 days post-fertilization (dpf)) through to adulthood (175 dpf). While direct age equivalence between zebrafish and humans is not definitive, this developmental window captures a critical period of substantial growth and physiological maturation, which is relevant across vertebrates, including humans. By doing so, we aim to clarify the implications of such dietary shifts on growth, metabolic health, and overall physiological development, providing evidence to inform future dietary recommendations and sustainable food production strategies.

The goal of our research was to conduct a comprehensive, longitudinal study to elucidate the effects of alternative proteins on the health and development of a model vertebrate, the zebrafish. We took the approach of completely replacing the typical animal-based protein diet criticized for its high economic and environmental costs[28] with pea, milk, wheat, and whey proteins. The expectation was that a complete change in diet would quickly reveal dietary performance differences and increase the likelihood of capturing any physiological and microbiome changes.

Materials and methods

Animals and experimental design

Tupfel long-fin zebrafish were grown and maintained in a recirculating rack system at the University of Alberta (AB, Canada); under 14 : 10 light : dark photoperiod[29] (Text S1). At 7 dpf, larvae were randomly distributed into ~3 L tanks and fed GEMMA Micro 75 (Skretting, Westbrook, ME, USA) and brine shrimp (Argentemia, Redmond, WA, USA) twice daily. At 30 dpf and over a period of four days, the larvae were randomly allocated to 6 L tanks (30 fish per tank) and assigned to one of six dietary treatments. Each dietary treatment group consisted of six replicate tanks randomly distributed across two racks, resulting in 36 tanks. We confirmed that rack placement did not influence fish survival or growth ($p > 0.05$ for all comparisons). Feeding of the alternative diets commenced at 30 dpf, with rations set at 5% of body mass daily (estimated from sibling, non-experimental fish). To evaluate the impact of alternative proteins, we assessed survival, growth, muscle development, metabolic gene expression, and gut microbiome composition (see Fig. S1 for an experimental overview). Experimental procedures were approved by the University of Alberta's Animal Care and Use Committee (AUP #3635).

Diets

Six different diets were produced in-house. The protein component included a salmon-based fishmeal control (West Coast Reduction Ltd, Edmonton, AB), grade 1 pea and grade 2 pea protein isolates (which differed in terms of amino acids and macronutrients, Table S1), milk protein (80% casein hydrolysate and 20% whey protein concentrate), a milk/pea 2 (50 : 50) protein blend, or a hydrolyzed wheat gluten/pea 2 (50 : 50) protein blend. Plant proteins were commercially sourced. Diets were formulated to be isocaloric, isonitrogenous and isoenergetic so that any performance differences between the diets could be attributed to dietary protein source. Total raw protein constituted ~42% of the diet mass (Table S1)—an amount optimized for maximum growth and protein retention in zebrafish.[30] All diets were supplemented with 2.5% vitamin and mineral premix (Table S2; Lot no. X2260, MP Biomedicals, CA, USA), and 0.7% betaine was added for palatability[31] (Table S3). Diet preparation and analysis details are in Text S2. Essential amino acid profiles are shown as a percentage of daily fish requirements[32] (Fig. 1A and B).

Feeding trials

Before committing to all diets, we began with a 40-day preliminary trial; the whey protein diet did not support growth and so was discontinued. Subsequent feeding trials began at 30 dpf (day 0, Fig. 1E), since natural mortalities predominantly occur within the first 30 days. Survivorship was determined by counting the number of fish present in each tank every 2–3 days until the study concluded at ~6 mo. Typically, mortality rates during the 30–60 dpf developmental period may be up to 20%,[33] and so our benchmark for survivorship was 80%. All diets successfully met this threshold at the 60-day mark. At the conclusion of the trials, zebrafish were euthanized by buffered tricaine methanesulfonate (MS-222; Syndell; Nanaimo, BC, Canada).

Growth rate assessment

Zebrafish total length (TL) was measured bi-weekly until the study's conclusion. TL was determined using photographs of each tank taken from above (ImageJ;[34] Fig. 1E). On the last day of the study, standard length (SL) measurements were obtained directly from euthanized fish before proceeding with tissue collection (see Fig. 1C).

Muscle development assessment

Skeletal muscle sections of ~3 mm were dissected from the caudal trunk, anterior to the caudal fin and used for histological (Fig. 2B). A total of 68 fish were analyzed, with two fish (one male, one female) randomly selected per tank. Each dietary treatment included 12 fish, except for the pea-1 and wheat/pea-2 groups ($n = 10$) due to early tank termination. See Text S3 for histology methods.

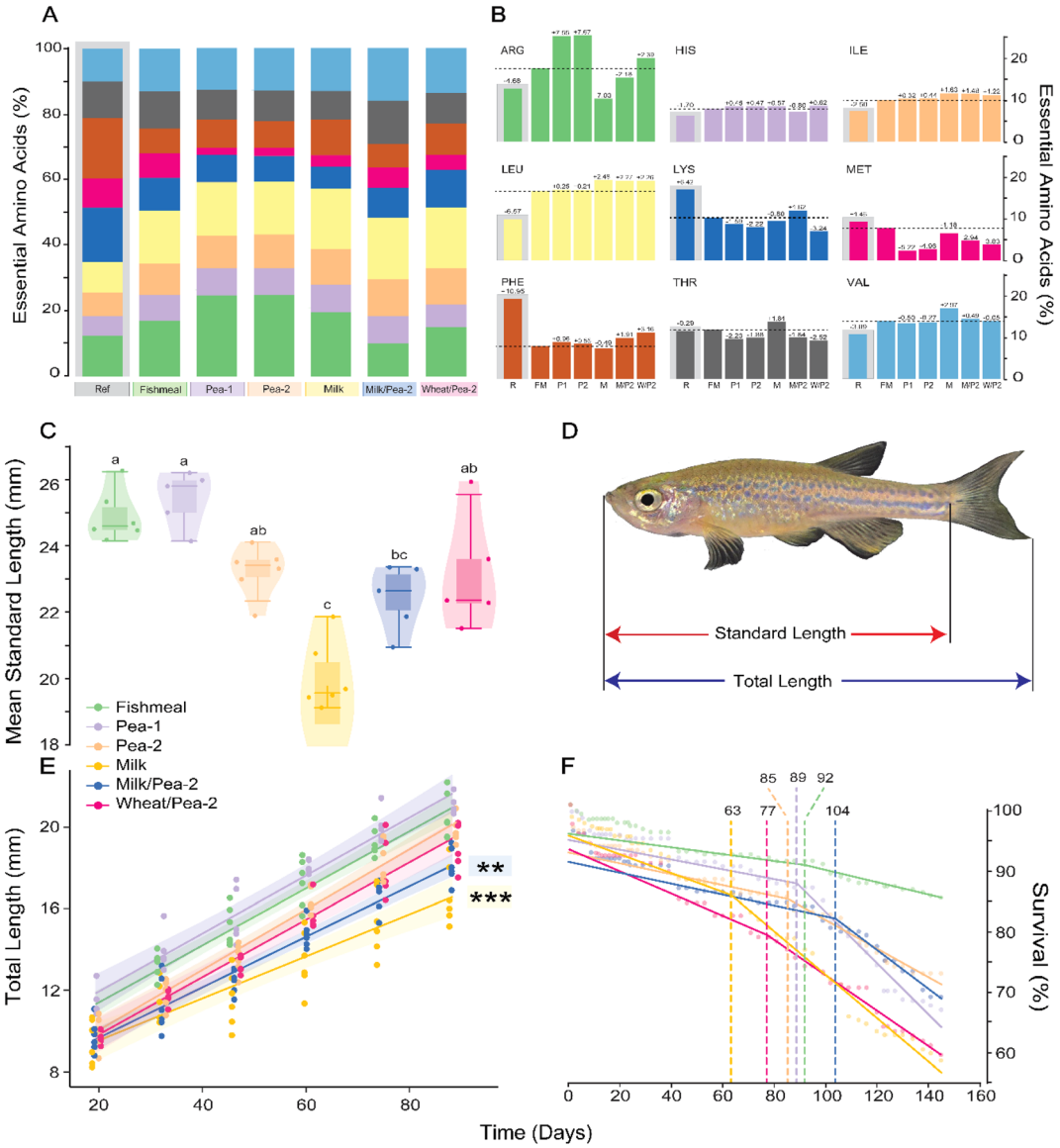

Fig. 1 Growth and survival of adult zebrafish on alternative protein diets. (A) Essential amino acid levels in the six diets expressed as a percentage of the daily requirement (*R*) for common carp (Hasan *et al.*[32]), a freshwater omnivorous fish with dietary requirements comparable to zebrafish. Colours represent specific amino acids and are consistent between (A) and (B). (B) The dashed line represents the fishmeal control diet, and numbers above bars indicate the percentage difference from the control. (C) Mean standard length at 175 dpf. Different letters denote significant differences between groups ($p < 0.001$). (D) Representative image of length measurements. (E) Total length (mm) over time with regression curves and standard error. Significance levels: $^{**}p < 0.01$, $^{***}p < 0.001$. (F) Segmented regression analysis of survival with breakpoints where survival rates shifted significantly ($p < 0.001$). All length measurements were taken from six fish per tank ($n = 6$ tanks for fishmeal, pea-2, milk, milk/pea-2; $n = 5$ tanks for pea-1 and wheat/pea-2).

Muscle cross-sectional areas were quantified (ImageJ), and perimeter adjustments were adjusted for accuracy. At least 150 distinct muscle fibers per fish were analyzed, sampling from epaxial and hypaxial regions to capture natural variation. Myonuclei counts were also assessed using the same images.

qPCR and 16s rRNA sequencing

Whole viscera were dissected from zebrafish immediately after euthanasia, snap-frozen in liquid nitrogen, and stored at −80 °C until either (1) RNA extraction using the TRIzol method (RNA extraction, cDNA synthesis, and qPCR procedures are available in Text S4; primers are available in Table S1), or (2) genomic DNA extraction, which was performed on ~10 mg of homogenized tissue utilizing the Gentra Puregene Tissue Kit (Qiagen; Hilden, Germany) as per the manufacturer's instructions. DNA extracts from two fish from the same tank (one male, one female) were pooled. Microbiome profiling was conducted on the isolated DNA samples by targeting the V4 variable region of the 16s rRNA gene *via* Illumina MiSeq sequencing, performed by

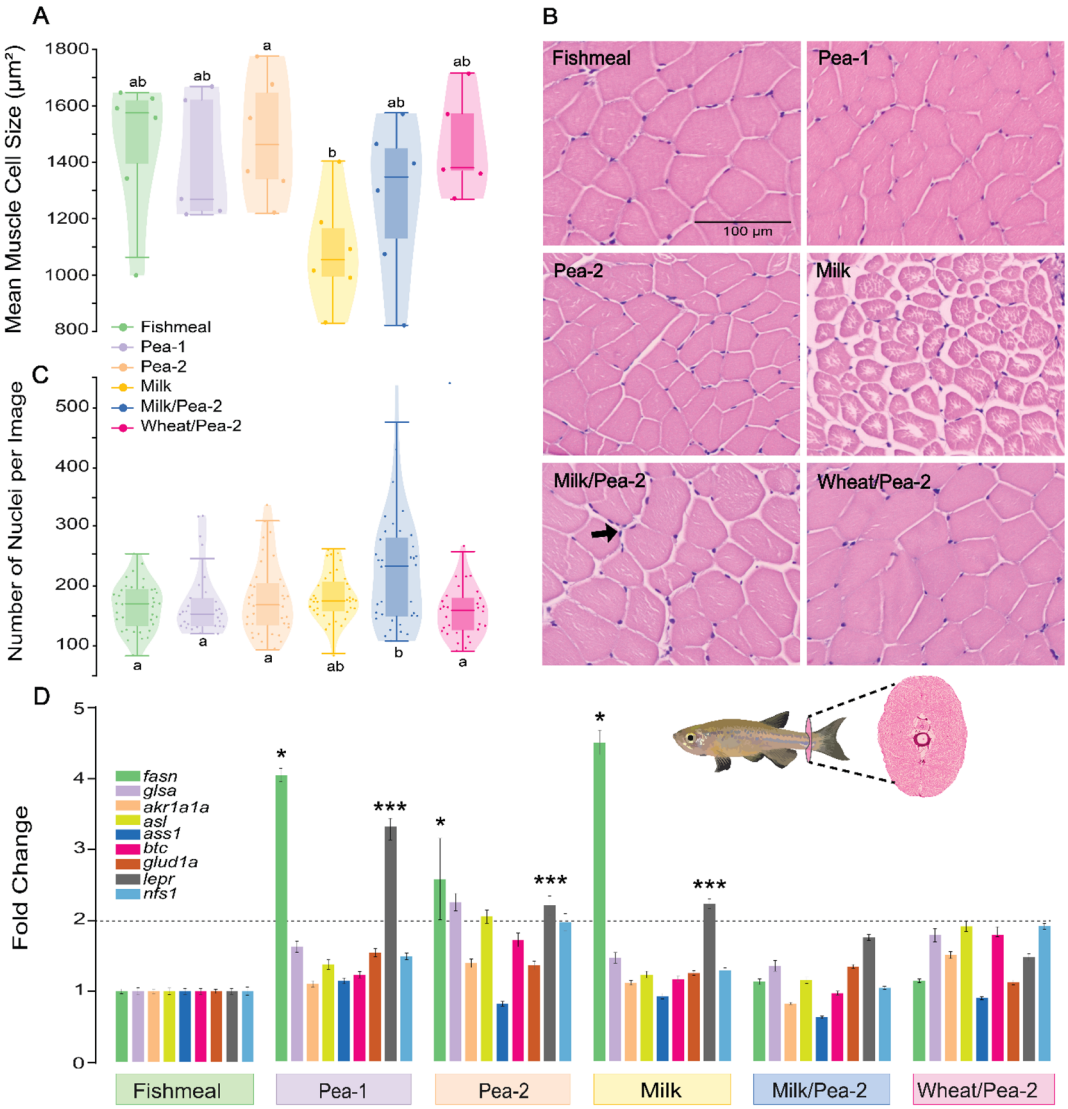

Fig. 2 Muscle development and gene expression of zebrafish reared on alternative protein diets. (A) and (C) Mean muscle fiber cross-sectional area (μm^2) and nuclei count. Outliers (above the 95th percentile or below the 5th percentile) were removed using boolean logic. Groups with different letters denote significant differences ($p < 0.05$). The horizontal line represents the median, the box represents the interquartile range, the whiskers denote the minimum and maximum values, and the violin aspect represents frequency. Data points were jittered for improved visualization. Sample sizes: two fish per tank, with n = 6 tanks (fishmeal, pea-2, milk, milk/pea-2) and n = 5 tanks (pea-1 and wheat/pea-2) per diet. (B) Representative hematoxylin and eosin-stained muscle sections from each diet, with myonuclei indicated by the black arrow, viewed under a compound light microscope at 20× magnification. The location of dissection in the caudal musculature and a representative cross-section spanning epaxial and hypaxial muscle fibers is shown. (D) Fold changes in the expression of metabolic genes in viscera tissue (*akr1a1a*, *asl*, *ass1*, *btc*, *fasn*, *glud1a*, *glsa*, *lepr*, and *nfs1*) were quantified and normalized to *eefla1l1* as an endogenous control. Each diet group comprised n = 10–16 biological replicates, with three technical replicates per biological replicate. Fold changes >2 (dashed line) or <0.5 were considered biologically significant. Statistical significance was as follows: *$p < 0.05$, ***$p < 0.001$. Error bars represent the 95% confidence interval.

Microbiome Insights (Richmond, BC, Canada). The complete procedure is in Text S5.

Statistical analysis

A segmented regression analysis with the Davies test was performed to detect breakpoints in zebrafish survival over time. Growth rate was modelled using multiple linear regression analysis (including "days since beginning dietary treatment" as a continuous predictor and "diet treatment" as a categorical predictor, with the fishmeal control diet assigned as the reference level). Muscle fiber area, myonuclei number, and Shannon diversity were evaluated using a one-way analysis of variance (ANOVA), followed by Tukey's HSD test. qPCR data was analyzed *via* the ΔΔCT method on the technical replicate data. Beta diversity was assessed with permutational multivariate analyses of variance (PERMANOVA) with treatment group as the main fixed factor and using 999 permutations for significance testing. Further detail of statistical analyses is

available in Text S6. All statistical analyses were performed in RStudio (RStudio; Boston, MA, USA) using R version 4.1.2.[35] Significance was accepted at $\alpha = 0.05$.

Results and discussion

Amino acid deficiency was linked to lower survivorship

The expectation was that the six diets would adequately support zebrafish growth and survival, and this was partially the case. Of the two pea protein based diets, pea-2 supported the highest survivorship among the alternative diets, with 74% of fish surviving the entire study duration (control was 88%; Fig. 1F). The discrepancy in survivorship between the animal-protein control and the alternative protein diets, particularly those containing pea protein, may be owe to deficiencies in specific amino acids. The pea-1 and pea-2 diets contained the lowest levels of methionine—an essential sulfur-containing amino acid abundant in eggs, meat, and seafood.[36] Although pea proteins are considered high-quality because they contain all essential amino acids, they are inherently deficient in sulfur-containing amino acids such as methionine and cysteine.[37] Methionine plays a vital role in homeostasis where it aids in the production of non-essential amino acids, including cysteine—a precursor for glutathione, a tripeptide involved in antioxidant defense.[36] Similar to our findings, diets deficient in methionine have been shown to reduce survival in juvenile grouper fish (*Epinephelus*)[38] and lake trout (*Salvelinus namaycush*).[39] Another study investigating dietary methionine deficiencies in mice demonstrated dysregulation in lipid metabolism, hepatic lipogenesis, cellular glutathione system deterioration, and oxidative stress induction.[40] Conditions such as these may be implicated in the pathogenesis of liver disease in model organisms and humans.[40]

The milk/pea-2 and pea-1 diets had survivorships of 70% and 68%, respectively. Survivorship in the milk and wheat/pea-2 diets differed markedly from this at 59% and 60%, respectively (Fig. 1F). These appreciable survivorship differences for the milk and wheat/pea-2 diets (40% *vs.* 30% in pea diets) suggest that these protein sources may have lacked the necessary components to sustain a model vertebrate through an extended developmental period.

Protein source determines survivorship

All diets enabled a majority of fish to reach the critical 60 d survivorship threshold, which captures a period that reflects growth into early adulthood (Fig. 1F). In various other animals, the quality of early life nutrition is made apparent in the health of the adults.[25,26] In our experiments, this reality emerged as decreases in survivorship after 60 d. We determined the breakpoints at which significant declines in survival occurred (92 ± 8.1 days, $p < 0.001$; $R^2_{adj} = 0.9658$), pea-1 diet (89 ± 2.4 days, $p < 0.001$, $R^2_{adj} = 0.9795$), and pea-2 diet (85 ± 4.9 days, $p < 0.001$, $R^2_{adj} = 0.9701$). The milk diet displayed the earliest breakpoint, with a decline in survival occurring at 63 ± 3.6 days ($p < 0.001$, $R^2_{adj} = 0.9908$). The abrupt decline in fish survival after the breakpoints suggests an inability of alternative protein sources to meet the nutritional demands of growing zebrafish after a particular developmental stage, which led to mortality *via* malnutrition.[41] Previous research on Indian major carp fry revealed that diets lacking essential nutrients can result in reduced survival and growth rates compared to fish fed nutritionally adequate diets.[42] Possible reasons for this inadequacy include a general imbalance in essential amino acids or other dietary components, along with differences in the bioavailability and digestibility among the protein sources.[43] Prolonged deficiencies in essential nutrients and amino acids are well-documented to have detrimental effects on health, including developmental abnormalities and mortality, a phenomenon observed across virtually all organisms.[44–46] Even acute periods of malnutrition experienced during critical developmental periods have been associated with impaired development and survival.[47] Previous research on larval zebrafish demonstrated that the severity of malnutrition directly correlated with the degree of observed mortality, impaired growth, swimming performance, and neurodevelopment.[48]

A particularly telling result was that the milk/pea-2 diet exhibited a survival comparable to pea-2 (70% *vs.* 74%). This suggests a robust compensatory effect of pea-2 in ameliorating the adverse outcomes observed with milk alone, possibly due to a lower lactose content and enhanced palatability, digestibility, and bioavailability. Additionally, the higher methionine content in the milk diet may have offset the methionine deficiency present in pea protein, resulting in a more balanced amino acid profile once combined (Fig. 1A). The wheat/pea-2 diet, which incorporated hydrolyzed wheat gluten with pea protein, resulted in a lower survival of 60%. While the pea-2 diet alone approached acceptable performance, the addition of wheat appeared to cause a decline in survival. This unrescuable decrease in survival may indicate the presence of adverse compounds in wheat, such as antinutritive factors, that diminished the overall nutritional quality of the protein. Antinutritive factors, including phytates, tannins, saponins, and protease inhibitors (*e.g.*, trypsin), can be found in plant-based sources like cereals and legumes,[49] reducing palatability, digestibility, and bioavailability of the protein.[50–52] Antinutritional factors can also induce intestinal inflammation and impair growth rate in fish, especially at high levels of dietary inclusion.[53–55] Moreover, during the processing of wheat proteins by manufacturers, heat and alkaline treatments can induce physicochemical changes in wheat gluten, forming oxidized amino acids and protein crosslinks.[50,56] These heat-mediated alterations can detrimentally affect protein digestibility and hinder the availability of amino acids in the protein.[50] While the presence and interactions of these compounds in our diets remain speculative, their potential impact should not be dismissed. Thus, it is conceivable that antinutritive factors affected the nutrient utilization of our zebrafish, potentially contributing to reductions in both survival and growth.

Pea proteins support somatic growth

In this study, growth—quantified as the increase in body length over time—was compared across dietary groups (Fig. 1C and E). We measured body length since it is a more reliable indicator of maturation stage than numerical age alone, as zebrafish development can be influenced by a multitude of factors (*e.g.*, temperature, water quality, and diet).[33] Body length varied over time among the dietary groups by as much as 120% (linear regression analysis revealed $F_{11,600} = 674.4$, $p < 0.001$, $R^2_{adj} = 0.924$; Fig. 1E). Zebrafish fed the plant-based pea-1, pea-2, and wheat/pea-2 diets achieved comparable sizes to the fishmeal control (25.9 ± 0.52, 26.6 ± 0.68, 25.2 ± 0.57, and 24.9 ± 0.51 mm, respectively), but those fed the milk ($p < 0.001$) and milk/pea-2 ($p < 0.001$) diets were approximately 10% smaller (22.6 ± 0.53 and 23.6 ± 0.48 mm, respectively).

Zebrafish fed milk-based diets exhibited the slowest growth rates, with the milk diet trailing the control by 0.04 ± 0.006 mm day^{-1} ($t = -7.178$, $p < 0.001$) and the milk/pea-2 diet by 0.02 ± 0.006 mm day^{-1} ($t = -3.097$, $p < 0.01$). Correspondingly, the final body lengths of adult fish at the end of the study were significantly reduced in the milk-based diet groups ($H_5 = 32.289$, $p < 0.001$; Fig. 1C). Additionally, the whey diet failed to support survival and growth during a 40-day preliminary trial, leading to its exclusion from further experiments. These findings are consistent with studies on Nile tilapia (*Oreochromis niloticus*) fingerlings, where high levels of fishmeal replacement with whey protein hindered growth.[57] However, low levels of whey protein inclusion were found to be tolerable.[57,58] Given the strong dependence of fish growth on the nutritional quality of their daily feed,[59] the absence of significant differences between our plant-based diets and the fishmeal control suggests that pea protein holds promise as a viable alternative for long-term sustenance.

The milk protein diet was the least favorable regarding survival and growth, resulting in the shortest body lengths and only 59% survival throughout the study period. This was surprising, as milk is considered a high-quality protein for supporting muscle health, weight management, and immune function.[60,61] It is plausible that this resulted from species-specific factors, where zebrafish found the milk proteins unpalatable (even though all diets were supplemented with betaine), or that they faced challenges in effectively metabolizing components of the milk diet. We posit these scenarios given that the milk diet lacked any notable essential amino acid deficiencies that could account for such significant reductions in growth and survival. It is worth noting that the milk protein comprised 80% casein hydrolysate and 20% whey protein. Casein hydrolysate contains minimal amounts of the milk disaccharide, lactose, compared to whey protein concentrates (which typically contain higher levels of lactose).[62–66] While zebrafish are not mammals, transcriptomics data indicates they still express low levels of lactase—the digestive enzyme required for breaking down lactose into usable energy sources—with the highest expression levels in the intestine.[67–69] The rapid mortality observed in fish fed the whey diet during the preliminary trial may be attributed to zebrafish's limited capacity to metabolize high amounts of lactose, given that milk is not a typical component of their diet (especially in such abundance). In humans, lactose intolerance symptoms (*e.g.*, abdominal pain, bloating, diarrhea) depend on the lactose dose and the extent of lactase expression in the intestine.[70] Even lactose-tolerant mammals have shown symptoms such as diarrhea, bloating, reduced growth rate, and premature death when exposed to overwhelmingly high levels of dietary lactose.[71,72] The milk diet—composed primarily of casein—likely outperformed the preliminary whey diet because its lower whey protein content reduced the overall lactose burden. In line with our observations, an earlier study using Nile Tilapia (*Oreochromis niloticus*), a freshwater omnivorous fish having similar protein requirements to zebrafish,[32] found that a diet combining fishmeal with whey protein of up to 28% inclusion permitted growth and development.[58] However, higher levels of whey inclusion were detrimental to fish health, causing cellular apoptosis and damage in intestinal and liver tissues.[58] Consequently, future investigations may benefit from exploring lower inclusion levels within this range rather than opting for complete replacements.

While we cannot rule out that food intake was an issue based on palatability, we did include betaine to specifically offset this.[31] Casein hydrolysate can have an unpleasant taste that some species may find aversive,[73,74] but whether this is true for fish is uncertain. Thus, these findings warrant the need for additional research to fully understand the suitability of milk-derived proteins for zebrafish and should not be generalized beyond this model without further evidence.

Pea protein diets sustain muscle health

Pea protein diets supported muscle development as cell sizes were approximately the same across diets (Fig. 2A). Similar results have been observed in juvenile lumpfish (*Cyclopterus lumpus*), where pea-based proteins performed comparably to fishmeal in promoting muscle fiber growth without compromising body mass.[75] This has tremendous implications for the uptake of pea protein as a sustainable substitute for conventional animal-based proteins, suggesting it can support muscle development without compromising growth. Early life nutrition not only influences immediate growth but also has lasting effects on physiological development, including muscle health extending into adulthood.[76] Muscle quality is commonly quantified by examining the cross-sectional area of muscle fibers, which is an indicator of muscle mass and hypertrophy,[77–79] along with the number and distribution of their myonuclei.[80,81]

A surprise was that zebrafish fed milk (and milk/pea-2) had fibers that were ~30% smaller ($p = 0.049$; Fig. 2A). This highlights the potential limitations of milk-based proteins in supporting zebrafish muscle development compared to plant-based or fishmeal-based alternatives.

In addition to fiber size, we looked at myonuclei, which support muscle maintenance and regulate protein synthesis.[79,80,82] Typically, an increased abundance of myonu-

clei is a positive indication of growth (hypertrophy).[81,82] In our study, fish that grew successfully (*i.e.* those on pea diets) had myonuclear counts comparable to the control (~150–170), reinforcing that pea protein supports muscle development as effectively as fishmeal.

As above, the milk diet's lack of support was apparent, in this case with an alteration in the number of myonuclei in the milk/pea-2 diet. Unlike the reduced cross-sectional area observed in diets containing milk, myonuclei numbers increased by ~30% (190–221; Fig. 2C). This elevation may indicate increased muscle damage, ongoing repair, and stress adaptation processes.[83] We suspect myonuclei in milk-fed fish migrated and clustered along myofibrils, with some infiltrating muscle fibers—an indicator of muscle stress pathology.[80] Considering cross-sectional area with myonuclei counts, the data indicate the milk and milk/pea-2 diets provided dissatisfactory nutrition. These findings suggest that the milk-derived proteins had lower bioavailability or digestibility needed for muscular maintenance, particularly in lactase-deficient organisms, making them less effective for muscle maintenance.

Diet modulates metabolic gene expression

Dietary composition influences gene expression by modulating mRNA stability and translation rates, directly affecting metabolic pathways with implications for health, development, and disease prevention.[84–86] In zebrafish, dietary composition affects a range of genes related to metabolism and growth-related pathways.[87] We noted the upregulation of fatty acid synthase (*fasn*), a lipogenic gene, in the milk diet (4.5 fold), pea-1 diet (4 fold), and pea-2 (2.5 fold) diets compared to control ($F_{5,74} = 2.387$, $P = 0.046$; Fig. 2D).

An upregulation of *fasn* correlates with a methionine deficiency and an arginine surplus.[40,88] Both pea-based diets contained an amino acid profile consistent with these nutritional imbalances relative to the fishmeal control. However, this does not fully explain the upregulation of *fasn* in the milk protein diet, which does not share these specific amino acid deficiencies. In humans, increased consumption of whey protein has been linked to enhanced insulin sensitivity through the upregulation of insulin receptor substrates (*e.g.*, *IRS-1* and *IRS-2*) and *fasn*, leading to improved hepatic glucose metabolism.[89] The upregulation of *fasn* presents ambiguous implications for human health holistically. Specifically, *fasn* upregulation is strongly associated with the development and progression of various cancers due to its role in dysregulating the FOXO3–FOXM1 axis and promoting fatty acid synthesis.[90] Alternately, because of its functional role in improving insulin sensitivity, *fasn* upregulation may benefit individuals with a genetic predisposition or multiple risk factors for diabetes.[89] Furthermore, *fasn* upregulation has also been hypothesized to increase insulin and chemotherapeutic resistance through signaling cascades in the PI3K–AKT pathway.[90,91] Overall, the observed increase in *fasn* expression in fish fed the pea diet suggests, at minimum, that this protein source evoked a different metabolic response than fishmeal. Further research could determine whether a change in *fasn* is adaptive and in response to dietary stress or is associated with any long-term, adverse health outcomes.

The leptin receptor gene (*lepr*), which regulates appetite and satiety,[92] increased 3.5-fold in the pea-1 diet and 2-fold in the pea-2 and milk diets ($F_{5,74} = 5.534$, $p < 0.001$; Fig. 2D). Since leptin receptors in intestinal microvilli influence gastric nutrient absorption, these findings suggest dietary proteins can modulate satiety signaling.[92] The leptin receptor plays a central role in regulating appetite, food intake, and body mass,[93] and is intrinsically linked to diabetes and various metabolic disorders, with the knockout of the *lepr* gene in mammals resulting in a diabetic phenotype characterized by morbid obesity.[94] Del Vecchio *et al.*[94] found that *lepr* impacts transcription in orexigenic pathways more than anorexigenic pathways in zebrafish, emphasizing its regulatory importance. Given the receptor's role in suppressing appetite and nutrient absorption, it is unsurprising that *lepr* was upregulated in the milk-fed fish, as this diet showed reduced growth and muscle cross-sectional area.

To understand the contribution of these genes to metabolism, we can consider their expression in conjunction with nutrient availability. The most notable instances of nutrient surplus were arginine in the pea protein diets and valine in the milk diet (Fig. 1A). Arginine is a crucial amino acid for insulin regulation, acting as an insulinotropin that promotes tissue absorption of nutrients and stimulates the release of glucagon.[95] Diets with excessive arginine levels are associated with increased volumes of subcutaneous adipose tissue, consistent with an upregulation of *fasn*.[96] Furthermore, valine surpluses are associated with increased hepatic fatty acid synthesis and *fasn* expression.[97] We suspect that the upregulation of both *fasn* and *lepr* are products of niche nutritional surpluses, with arginine driving this response in the pea-based diets and valine in the milk diet. The functional role of *fasn* is the production of fatty acid synthase to facilitate increased fat storage during nutrient excess, which, in turn, elevates adipose tissue and leptin secretion.[98,99] As such, upregulation of both *fasn* and *lepr* is a rational physiological response to the amino acid imbalances in these diets and sheds light on the importance of a balanced amino acid profile when investigating the suitability of alternative protein sources. Importantly, the lack of differential expression in other metabolic genes indicates that, overall, the alternative protein diets did not induce major negative changes in the genetic profile of the zebrafish relative to the control. This highlights the potential viability of these alternative diets, provided they are carefully formulated to maintain a balanced amino acid profile.

Of the other genes we examined, glutaminase a (*glsa*), argininosuccinate lyase (*asl*), and cysteine desulfurase (*nfs1*) (genes involved in the metabolism of glutamine, the urea synthesis pathway and cysteine decomposition, respectively[100–103]), showed a slight 2-fold upregulation in the pea-2 diet. Conversely, no notable changes were observed in the genes for aldo-keto reductase family 1 (*akr1a1a*), argininosuccinate synthase 1 (*ass1*), betacellulin (*btc*), or glutamate dehydrogenase 1a (*glud1a*). The upregulated genes serve as

markers indicating that alternative protein diets can selectively influence key metabolic pathways, and so have implications for optimizing proteins formulations to enhance metabolic health and growth.

Alternative proteins remodel the gut microbiota

Gut microbiota are recognized as key regulators of health that influence host nutrition[104] and metabolism.[105] Across hosts, microbiota composition can vary drastically and be shaped by genetics, environment, and diet.[105] In our study, we aimed to isolate the effects of diet by using genetically ~identical individuals held in a constant environment.

Zebrafish are increasingly used as a model for gut microbiota research because their core gut bacterial community shares substantial similarities with that of humans,[106] including dominant groups such as Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria.[107,108] These conserved taxa are functionally important for nutrient metabolism[109] and gut homeostasis,[106] supporting the translational relevance of zebrafish studies.

The richness and evenness of alpha or within-individual diversity can be scored using the Shannon index. A high Shannon index indicates functional redundancy within the microbial community that provides stability and resilience to the microbiome, and thus benefits host health.[110,111] Across diets, Shannon diversity was similar ($F_{5,27}$ = 2.509, P = 0.055), but highest in fish fed the pea-1 (2.37 ± 0.36) and pea-2 (1.52 ± 0.48) diets (Fig. 3A), and lowest in fish fed the fishmeal (1.15 ± 0.32). These data suggest a trend toward greater microbial health within the pea-fed groups.

Differences in microbiota across individuals are apparent in the beta diversity, which can be summarized into Bray–Curtis dissimilarities. We performed a PCoA on the Bray–Curtis dissimilarities to visualize beta diversity between diets. There was a pronounced separation between microbial communities for fish fed the alternative diets and those fed the fishmeal control (Fig. 3C; R^2 = 0.4958, p = 0.001). Specifically, all diets differed from the fishmeal control (fishmeal & pea-1, R^2 = 0.576, p = 0.013; fishmeal & pea-2, R^2 = 0.810, p = 0.013; fishmeal & wheat/pea-2, R^2 = 0.660, p = 0.013; fishmeal & milk, R^2 = 0.688, p = 0.013; fishmeal & milk/pea-2, R^2 = 0.545, p =

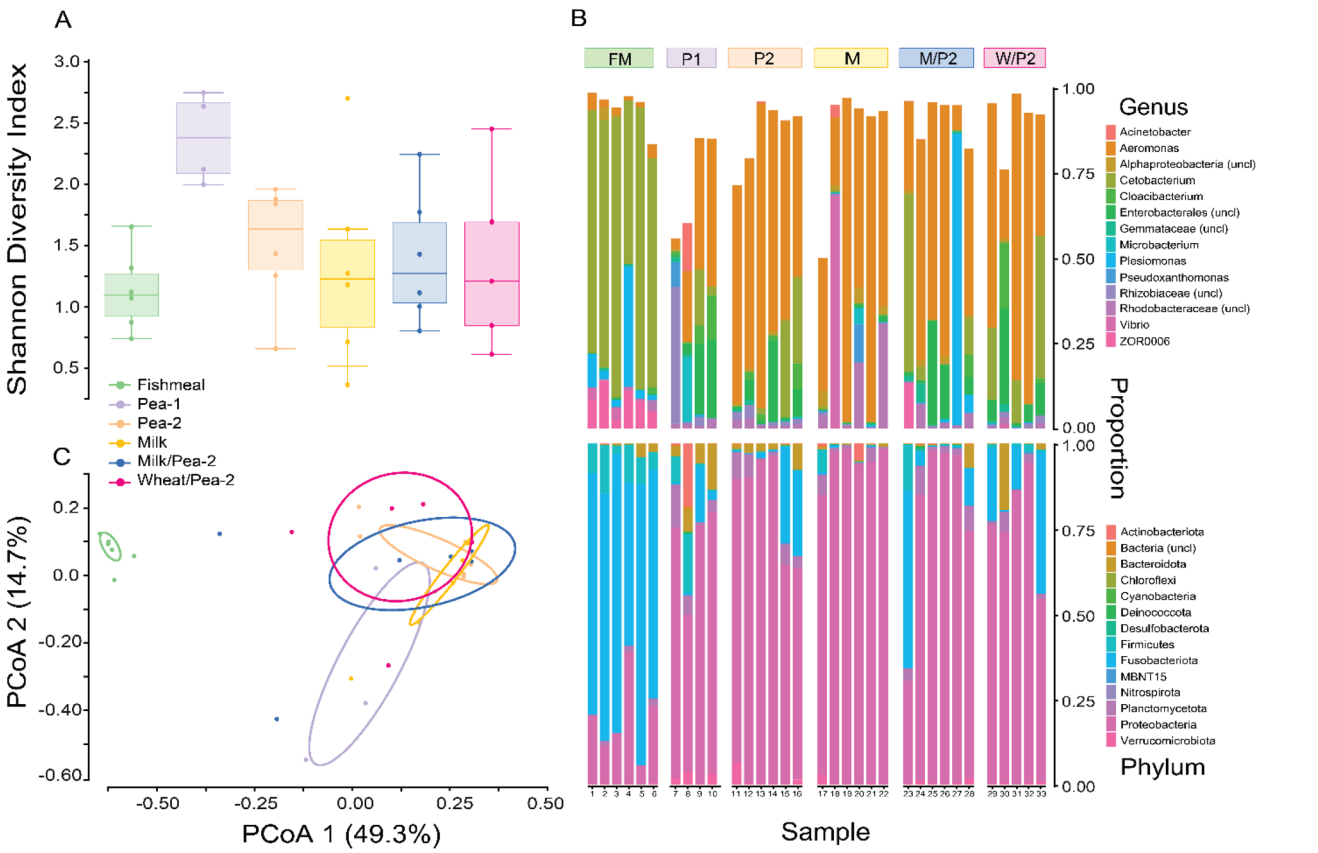

Fig. 3 Microbiome diversity of zebrafish reared on alternative protein diets. (A) Alpha diversity, measured using the Shannon diversity index. No significant differences in alpha diversity were observed across diet groups (p = 0.055). (B) Beta diversity visualized using a PCoA ordination plot of Bray–Curtis dissimilarities. Axes represent the percentage of variation explained by the first two principal coordinates (PC1 and PC2). (C) Taxonomic composition of dominant bacterial taxa at the phylum and genus levels, displayed as stacked bar plots. Each bar represents pooled DNA from two fish (one male, one female) per tank. Diets included fishmeal control (FM; n = 6), pea-1 (P1; n = 4), pea-2 (P2; n = 6), milk (M; n = 6), milk/pea-2 (M/P2; n = 6), and wheat/pea-2 (W/P2; n = 5). Unfilled portions of the bars represent less abundant taxa.

0.013), and the two pea proteins differed from each other, R^2 = 0.253, p = 0.013.

The taxonomic rank of microbiota were visualized at the phylum and genus level, and the relative abundance of the most common taxa were compared across diets (Fig. 3B).

Previous studies on zebrafish found the gut microbiome was dominated by Proteobacteria (~40–60%), Firmicutes (~10–25%), Fusobacteria (~5–15%), Actinobacteria (~1–25%), and Bacteroidetes (~2–10%).[112–115] In our study, zebrafish fed the alternative diets more closely resembled the above studies compared to our control, with ~68–94% Proteobacteria (control ~19%), ~0.4–7% Firmicutes (control 8%), ~0.3–15% Fusobacteria (control 70%), ~0.02–5% Actinobacteria (control 0.03%), and ~0.4–7% Bacteroidetes (control 1%). A decrease in Proteobacteria and an increase in Firmicutes has been seen in zebrafish microbiomes altered by exposure to dysbiotic agents.[112]

Across our diets, taxonomic analysis of the gut microbiota's relative abundance revealed variations at all levels—appearing markedly different across the diet groups. While Fusobacteriota predominated in the fishmeal control, the other experimental diets were associated with Proteobacteria (Fig. 3B). At the genus level, the fishmeal diet was dominated by *Cetobacterium*, whereas the alternative diets were characterized by a higher abundance of *Aeromonas* (Fig. 3B). Functionally, the proportion of Aeromonas was negatively associated with survivorship (R^2 = 0.535, $F_{2,6}$ = 221.6, P < 0.0001), whereas Cetobacterium abundance showed a positive association with survivorship (R^2 = 0.709, $F_{2,6}$ = 354.8, P < 0.0001).

A new finding, and one worthy of further discussion, is the presence of Planctomycetes in all diets (~1–6% Planctomycetes). The role of Planctomycetes is not fully understood. Some studies find that Planctomycetes behave as an opportunistic pathogen in zebrafish intestines, and therefore conclude that their presence is associated with diseased gut microbiomes.[116] Others hypothesize that Planctomycetes play specific roles in the functioning of the teleost gut microbiome, such as the breakdown of sulfated polysaccharides by heterotrophs or the cycling of nitrogen by anaerobes.[117] The presence of Planctomycetes in this study warrants further investigation to clarify whether they contribute to gut health or reflect pathological changes, particularly in the context of these alternative protein diets.

Conclusion

Alternative proteins are required to meet global protein demand. The long-term health implications of switching to diets solely based on alternative proteins remains largely unknown. We found that some common alternative proteins, including pea, whey and wheat, differed in their abilities to support the development and health of a model vertebrate. Of the proteins tested, pea, with supplementation, could serve as a complete replacement for animal protein. This protein supported healthy growth and muscle development, and it appeared that the microbiome remodeled itself in response to meet the atypical source. Overall, this study suggests that humans have both the protein and microbiome necessary to transition away to an animal protein-free diet, within one generation.

Author contributions

Conceptualization: DM, KT; Data curation: CN; Formal analysis: CN, ET; Funding acquisition: KT, DM; Investigation: AB, AS, AN, CN, CS, JL; Methodology: AB, CN; Project administration: DM, KT; Supervision: KT; Visualization: MS, LF, CN; Writing – original draft: CN; Writing – review & editing: AB, AN, CS, DM, KT.

Conflicts of interest

There are no conflicts to declare.

Abbreviations

dpf	Days post-fertilization
OTU	Operational taxonomic unit
qPCR	Quantitative polymerase chain reaction
RQ	Relative quantification
SL	Standard length
TL	Total length

Data availability

Data is available through Dryad https://doi.org/10.5061/dryad.v15dv427m.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d5fo01990g.

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