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Optimizing winged-bean meal through oven and autoclave heating as viable alternative for plant-based aquafeed

Optimasi pembuatan tepung kacang kecipir melalui pemanasan dengan oven dan autoclave sebagai alternatif potensial untuk pakan ikan berbasis nabati

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ABSTRACT
Winged-bean meal can serve as a substitute for soybean meal in plant-based fish feed, despite its trypsin-inhibitor properties, which can be alleviated through heating processes. This study aimed to explore the potential of winged-bean meal subjected to different heating methods, namely oven (110 °C for 30 minutes) and autoclave (121 °C for 30 minutes), based on proximate analysis and trypsin activity. The study applied three treatments with four replications: oven heating (O), autoclave heating (A), and control (without heating, E). All data were analyzed using analysis of variance and Duncan's multiple range test to determine the best treatment. Data were also compared to the nutrient requirements of several cultured freshwater fish in Indonesia. Heating treatments improved *in vitro* trypsin activity (1300 units/O and 1135 units/A vs. 835 units) but reduced protein (29.05±1.96%/A and 31.73±1.52%/O vs. 35.17±0.38%) and moisture contents. Additionally, the O treatment met the standards for aquafeed protein content (27-45%) and potentially fulfilled the protein requirements for catfish (25-40%), Nile tilapia (30-35%), and Java barb (30-32%). Therefore, oven heating presents a viable candidate as an alternative plant-based aquafeed ingredient. Nevertheless, the in vivo application of winged-bean meal as an aquafeed ingredient should be further evaluated to determine whether heating treatment effectively enhances digestive activity, nutrient retention, and growth performance in target fish species.

ABSTRAK
Tepung kacang kecipir dapat digunakan sebagai pengganti tepung kedelai dalam pakan ikan berbasis nabati, meskipun memiliki sifat penghambat tripsin yang dapat dikurangi melalui proses pemanasan. Penelitian ini bertujuan untuk mengkaji potensi tepung kacang kecipir dengan metode pemanasan yang berbeda, yaitu oven (110 °C selama 30 menit) dan autoclave (121 °C selama 30 menit), berdasarkan analisis proksimat dan aktivitas tripsin. Penelitian ini menggunakan tiga perlakuan dengan empat ulangan, yaitu pemanasan dengan oven (O), autoclave (A), dan kontrol (tanpa pemanasan, E). Semua data dianalisis menggunakan analisis varian dan uji rentang berganda Duncan untuk menentukan perlakuan terbaik. Data juga dibandingkan dengan kebutuhan nutrisi untuk beberapa ikan air tawar yang dibudidayakan di Indonesia. Perlakuan pemanasan dapat meningkatkan aktivitas tripsin *in vitro* (1300 unit/O dan 1135 unit/A vs 835 unit), namun mengurangi kandungan protein (29.05±1.96%/A dan 31.73±1.52%/O vs 35.17±0.38) dan kelembaban. Selain itu, perlakuan O memenuhi standar bahan protein pakan ikan (27-45%) dan dapat memenuhi kebutuhan protein untuk ikan lele (25-40%), nila (30-35%), dan tawes (30-32%). Oleh karena itu, pemanasan dengan oven merupakan kandidat yang tepat sebagai bahan pakan ikan berbasis nabati alternatif. Namun demikian, aplikasi in vivo tepung kacang kecipir sebagai bahan pakan ikan perlu dievaluasi lebih lanjut, untuk mengetahui apakah perlakuan pemanasan efektif dalam meningkatkan aktivitas pencernaan, retensi nutrisi, dan pertumbuhan ikan target.

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INTRODUCTION

Feed is a crucial component of aquaculture operations, accounting for 70-80% of total operational costs (Tartila, 2023), and the rising cost of feed ingredients, such as soybean meal—which is widely used in the food and feed sectors due to its high protein content and favorable amino acid profile (Sriwichai et al., 2021)—significantly impacts the profitability and sustainability of aquaculture, especially in regions like Indonesia that rely on imports to meet their soybean meal demand; as the global demand for aquafeed continues to grow, there is an urgent need to identify alternative, cost-effective feed ingredients that can reduce dependency on imported soybean meal, a challenge that has prompted researchers and industry stakeholders to explore alternative plant-based protein sources that can be locally produced and are economically viable.

One promising alternative is winged-bean meal, derived from the winged bean (*Psophocarpus tetragonolobus*), a leguminous plant belonging to the Fabaceae family. Winged-bean meal has been recognized for its high protein content, which ranges between 36-37%, comparable to the protein content in soybean meal, which ranges from 35-40% (Adegboyega et al., 2019; Ibáñez et al., 2020). Moreover, winged beans are known for their adaptability to various climatic conditions, making them a potentially sustainable and locally available feed ingredient (Singh & Chauhan, 2023). However, a significant challenge in using winged-bean meal in aquafeed is the presence of anti-nutritional factors, particularly protease inhibitors such as trypsin inhibitors (Tanzi et al., 2019). Protease inhibitors can impair the digestive processes in fish by inhibiting proteolytic enzymes like trypsin and chymotrypsin, leading to reduced protein digestibility and nutrient absorption (Mohanty et al., 2020). This can negatively affect the growth performance and overall health of the fish.

Various methods have been explored to mitigate the anti-nutritional effects of protease inhibitors in plant-based feed ingredients. These methods include thermal processing (heating), soaking, fermentation, and enzymatic treatments (Bepary et al., 2023). Among these, thermal processing is particularly effective in reducing protease inhibitor activity because it denatures the protein structures of the inhibitors, rendering them inactive (Saadi et al., 2022). Heating can be applied using different techniques, such as oven roasting and autoclaving. Oven roasting involves dry heating at temperatures around 110°C for 30 minutes, while autoclaving uses hydro-pressure heating at 121°C for 30 minutes (Qin et al., 2022). These methods have been shown to significantly reduce the levels of protease inhibitors in soybean meal, with reductions ranging from 30-100% (Sá et al., 2020). The application of similar heating methods to winged-bean meal could potentially enhance its suitability as an aquafeed ingredient by improving its protein digestibility and nutritional value.

In ruminants, winged bean tubers have been applied as an alternative feed ingredient that could replace corn meal (Suntara et al., 2023; Srichompoo et al., 2024). Meanwhile, winged beans as feed additives have also been approved to increase the gonad maturity level of giant gourami (*Osphronemus gouramy*) (Febnikayani et al., 2018) and African catfish (*Clarias gariepinus*) (Tondang et al., 2019). For heated winged-bean meal application as main feed ingredient in aquafeed, Fagbenro (1999) reported that African catfish fed with heated winged-bean meal showed higher growth performance and protein digestibility compared to those fed with non-heated meal. However, most of these studies have focused on in vivo assessments without providing a detailed understanding of the underlying biochemical changes, particularly regarding protease (trypsin) activity and the specific nutrient requirements of different fish species (*in vitro* studies). This gap in the literature highlights the need for comprehensive studies that not only evaluate the overall performance of fish fed with heated winged-bean meal but also investigate the specific biochemical mechanisms and nutrient profiles that contribute to these outcomes. Understanding the changes in trypsin activity and nutrient composition after thermal processing is crucial for optimizing the use of winged-bean meal in aquafeed formulations.

Therefore, this study aims to bridge this gap by conducting a detailed investigation of the effects of two heating methods—oven roasting at 110°C for 30 minutes and autoclaving at 121°C for 30 minutes—on the protease inhibitor content, trypsin activity, and proximate composition of winged-bean meal. By analyzing these parameters, we seek to confirm whether these heating methods can effectively reduce protease inhibitor levels an d improve the nutritional quality of winged-bean meal. Additionally, this study will compare the nutrient profiles of heated winged-bean meal with the dietary requirements of several freshwater fish species commonly cultured in Indonesia, such as catfish (*Clarias* sp.), Nile tilapia (*Oreochromis niloticus*), and Java barb (*Barbonymus gonionotus*). By aligning the nutritional content of winged-bean meal with the specific needs of these fish species, we aim to establish its viability as an alternative plant-based aquafeed ingredient.

MATERIALS & METHODS

Materials and instruments

In this study, the primary sample used was winged-bean meal obtained from commercial sources. The reagents used for proximate analysis included H_2SO_4 95% (Sigma Aldrich Inc., USA), pellet NaOH 98% (Merck, USA), methyl red indicator (Sigma Aldrich Inc., USA), N-hexane 95% (Merck, USA), Whatman's filter paper No. 93 (Cytiva Inc., USA), and acetone 95% (Merck, Maryland, USA). For in vitro trypsin analysis, the materials used were trypsin powder, TCA 20% (trichloroacetic acid), NaOH 0.5 M, and Folin-Ciocâlteu's phenol reagent (Sigma Aldrich Inc., USA).

The instruments used for proximate analysis included a desiccator with a 2.4 L capacity (Pyrex, Japan), a heat-drying oven (240 V; 50/60 Hz, Thermo-Fisher Scientific Inc., Massachusetts, USA), a premium large muffle furnace (240 V; 50/60 Hz, Thermo-Fisher Scientific Inc., Massachusetts, USA), Kjeldahl flasks (PT. Iwaki Glass, Sumedang, Indonesia), and Soxhlet flasks (PT. Iwaki Glass, Sumedang, Indonesia). For in vitro trypsin analysis, the instruments used included a vortex mixer (Scilogex SCI-VS, Connecticut, USA), an incubator (Memmert GmbH IN110, Germany), and an E1000-UV spectrophotometer (PEAKE1000v-1, USA).

Experimental design

This study used an experimental method with a completely randomized design, that contains three treatments and four replications. The three treatments were composed of two different heating methods and a control treatment, namely winged-bean meal heated in an oven at 110 °C for 30 minutes (O), an autoclave at 121 °C for 30 minutes (A), and control (without heating, E), based on Samtiya et al. (2020).

Procedures

<u>Winged-bean meal production</u>

A sack of winged-beans with the weight of 10 kg were obtained from the wholesalers in Sukoharjo, Central Java, Indonesia. The winged-beans were separated from their pods manually, cleaned, and dried under the sunlight. The dried winged-beans were then separated on different containers for heating process with an oven at 110 °C for 30 minutes and an autoclave 121 °C for 30 minutes (Samtiya et al., 2020). After the heating process, the winged-beans were cooled at room temperature. After cooling, the winged-beans were crushed, grinded, and sieved to become a proper winged-bean meal. The winged-bean meal was preserved in a dried container at room temperature for further analysis.

<u>Proximate analysis</u>

As the winged-bean meal produced was unprocessed chemically, the proximate analysis was performed directly, after the production. The proximate analysis was conducted to determine the moisture, ash, crude fibre, crude protein, and crude fat contents of each winged-bean meal, after different heating treatments. The proximate analysis was conducted following the AOAC (2015) method. Crude lipid content was determined using the Soxhlet extraction method with Whatman's filter paper No. 93, Soxhlet flasks, heat-drying oven, and n-hexane 95% reagent. Crude protein content was determined with the Kjeldahl method with H_2SO_4 95%, NaOH 0.3 N (dissolved from pellet NaOH 98%), methyl red colouring-agent. Moisture content was determined by heating the sample in the heat-drying oven at 100-110°C for an hour. Ash content was confirmed by burning the sample at 600 °C for 4-6 hours with a premium large muffle furnace. Crude fibre was determined using the gravimetry method after chemical digestion with H_2SO_4 0.05 N (dissolved from

H_2SO_4 95%), NaOH 0.05 N (dissolved from pellet NaOH 98%), acetone 95%, Whatman's filter paper No. 93, heat-drying oven, and premium large muffle furnace.

Trypsin activity (*in vitro* analysis)

The *in vitro* analysis of trypsin activity was conducted, following the Anson method. Twelve units of reaction tubes were prepared and placed randomly in a rack, following the experimental design (three treatments × four replications). Before the test, the winged-bean meal from different heating methods and control treatment was dissolved with a distilled water and sieved to obtain 5 ml filtrate for each unit. Then, the filtrate was distributed into the reaction tubes and pre-incubated for 5 minutes at 37 °C.

Furthermore, each tube was added with 2 ml of trypsin enzyme solution (400 mg. L^{-1} phosphate buffer solution, pH 7.8), mixed homogenously with vortex, and incubated for 20 minutes at 37 °C. Incubation process was terminated exactly at 20 minutes after enzyme addition. To terminate the reaction, the 3 ml of 20% TCA (trichloroacetic acid, w/v) was added and mixed homogenously with vortex. Each tube was then moved in an ice water for 30 minutes, before moving the mixture from tube to a microtube for the centrifugation process at 1000×*g* in 15 minutes.

After the centrifugation process, the filtrate (solution) was taken at 2 ml and added with 4 ml of NaOH 0.5 M. This mixture was stood for 10 minutes and determined its absorbance at 650 nm wavelength with spectrophotometer. For blank samples, two reaction tubes were filled with 2 ml phosphate buffer solution (pH 7.8), 3 ml of 20% TCA, and 5 ml of distilled water, then mixed homogenously with vortex. The mixture was added with 1 ml of Folin-Ciocâlteu's phenol reagent and stood for 10 minutes, before the absorbance measurement with spectrophotometer at 650 nm wavelength. The absorbance value was calculated with the formula: Trypsin (unit) = (At – Ao)/ (0.001 × t), where At is the absorbance value after 10 minutes, Ao is the absorbance value before 10 minutes (initial time), and t is the incubation time (20 minutes).

Data analysis

The data collected (proximate analysis and trypsin activity) were confirmed firstly for their normality and homogeneity with a variance test. After the data were confirmed to be normally-distributed and homogenous, statistical analysis was applied using the one-way analysis of variance (one-way ANOVA) with *Microsoft Office Excel 2021* (*Microsoft Corp., Washington, USA*) and *SPSS ver. 16.0* (*IBM Corp., New York, USA*) software at 95% confidence level. When the data indicate a significant different ($p<0.05$), a further analysis, called the Duncan's multiple range test (DMRT) was applied to determine which treatment obtained the best values, according to the proximate analysis and trypsin activity. Data were also analysed descriptively by comparing the proximate analysis data with the nutrient requirements standard of several cultured freshwater fish to gain a better decision which treatment provides an opportunity for the winged-bean meal as potential plant-based aquafeed ingredient.

RESULTS & DISCUSSIONS

Proximate analysis

After the proximate analysis was performed, several parameters were significantly different among the treatments, namely moisture, crude protein, and nitrogen-free extract (NFE) contents. Other results were also present and described in Table 1. In Table 1, all proximate analysis results indicate an insignificant different value, except in the moisture, crude protein, and NFE contents. Also, all data followed the standard, reported by Bepary et al. (2023), except the moisture content. The moisture content in this study indicates that the winged-bean meal samples were thought to be initially dry and exceeded the harvesting period. Nevertheless, according to Putra et al. (2017), the plant-based aquafeed ingredient should maximumly contain moisture content of 12-13%, so the moisture contents of winged-bean meal in control and heating treatments are still qualified as aquafeed ingredient. Heating treatment could significantly reduce the moisture content in winged-bean meal, which means that the heating process is an effective method to decrease the moisture content, whereas a higher temperature and a longer cooking time causes a more significant moisture content reduction

Ahn et al., (2014). In addition, evaporation that occurs during the heating process at 100 °C causes the liquid component of the material to evaporate massively (Adawyah et al., 2020). A lower moisture content can actually prolong the preservation period of the feed ingredient without any fungi that grow on it.

Table 1. Proximate analysis of unprocessed winged-bean meal after different heating methods

Parameter	E (%)	O (%)	A (%)	Standard (%) ^	Soybean meal (%) #
Moisture	3.15±0.13[c]	2.40±0.42[b]	1.36±0.13[a]	5.30-12.58	5.60-11.50
Ash	4.71±0.67	4.40±0.21	4.45±0.13	3.52-4.90	4.50-6.50
Crude Fiber	16.65±4.63	15.30±4.95	12.55±1.85	1.55-26.15	4.32-34.30
Crude Protein	35.17±0.38[c]	31.73±1.52[b]	29.05±1.96[a]	27.20-45.02	31.00-47.00
Crude Fat	10.92±2.95	12.24±4.82	10.14±3.80	15.20-23.35	10.00-24.70
NFE*	29.41±6.33[a]	33.93±6.59[ab]	42.45±2.75[b]	14.20-35.66	31.70-37.85
GE**	1692.67±19.94[a]	1770.26±14.97[ab]	1883.43±17.76[b]	n/a	n/a

Note: n/a = non-defined data. E = unheated winged-bean meal (control), O = oven-heated winged-bean meal, A = autoclave-heated winged-bean meal, *NFE = Nitrogen-free extract, **GE = Gross energy (kJ/g) = (crude protein × 23.4 kJ/g) + (crude fat × 38.5 kJ/g) + (NFE × 17.2 kJ/g), according to Takeuchi et al. (2002), ^ = Standards according to Bepary et al. (2023). #Soybean meal nutrient contents reported by Ibáñez et al. (2020) as comparative quality properties of winged-bean meal after heating process. All values are presented as average and standard deviation values. Different superscript letters on the same line show a significant different value (DMRT, $p<0.05$).

Furthermore, the protein contents in all treatments present a significantly different value and followed the standard reported by Bepary et al. (2023). The O treatment had a protein content that was significantly higher than the A treatment, but the highest protein content was obtained from the E treatment. Although showing the declined values, the protein content results in different heating treatments were proportional to Adegboyega et al. (2019), who reported that the unprocessed winged-bean protein content from 25 plant varieties was among 28-31%. Therefore, the winged-bean meal after different heating treatments is still qualified as an alternative plant-based aquafeed ingredient for protein sources, that can be slightly similar to soybean meal, which contains 35-47% protein (Ibáñez et al., 2020). The declined values in heating treatments were occurred as heating caused denaturation process and protein breakdown (Qin et al., 2022). However, in economical approach, the non-heated winged-bean meal is considerable as a plant-based ingredient in aquafeed due to high protein content, closed to soybean meal.

In NFE contents, the E and A treatments were significantly different, but the O treatment was insignificantly different between both treatments. The highest NFE content was found in the A treatment, which means that the winged-bean meal after autoclave-heating produces high energy level, specifically the storage energy, as shown in the GE contents. A higher storage energy can cause the utilization of energy becomes difficult, as NFE is carbohydrates that requires to be altered as glycogen, if used for energy (Damayanti & Sjofjan, 2022). The A treatment also showed a higher NFE content than the standard value reported by (Bepary et al., 2023). Also, Putra et al. (2017) stated that a qualified NFE content for aquafeed ingredient is among 30-40%. This condition was occurred as autoclaving can highly break the cell wall structure of the bean, releasing the carbohydrates from the fiber as the main composition of the cell wall in plants (Damayanti & Sjofjan, 2022). Therefore, the winged-bean meal with oven-heating treatment (O) and non-heating treatment (control, E) are good for further usage as an aquafeed ingredient.

When compared to the soybean meal nutrient contents, heating process with an oven at 110 °C for 30 minutes (O) could produce a winged-bean meal that contained nutrients as same as presented in soybean meal. Meanwhile, the autoclaving could cause a lower protein content and higher NFE content, that are apart from the soybean meal nutrient contents.

Therefore, the O treatment is an appropriate method to produce winged-bean meal as an alternative replacement for soybean meal as the main plant-based ingredient in aquafeed formulation.

Trypsin activity

Heating treatments, either with oven or autoclave, could significantly affect the trypsin activity, following the *in vitro* observation. A detailed value of trypsin activity is presented in Fig. 1.

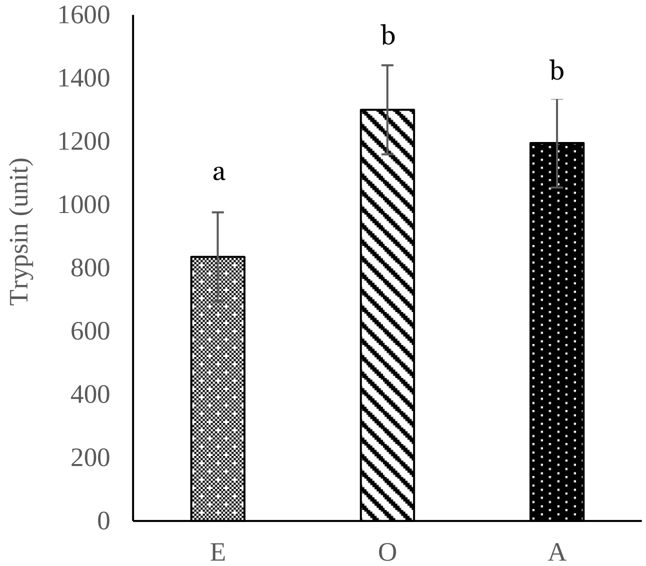

Figure 1. Trypsin activity, after being exposed to winged-bean meal that has been heated with different methods. Note: E = unheated winged-bean meal (control), O = oven-heated winged-bean meal, A = autoclave-heated winged-bean meal. All values are presented as average and standard deviation values. Different letters show a significant different value (DMRT, $p<0.05$).

Trypsin activity, as one of the protease enzymes found in fish, was found significantly higher in the heating treatments than in the control treatment. This condition indicates that heating process, both using oven (roasting) or autoclave (hydro-pressure heating), reduces the protease-inhibitor activity in winged-bean meal, as stated by (Samtiya et al., 2020). This study results were also similar to Saadi et al. (2022), who reported that heat-irradiation and heat-ionization in winged-beans could reduce the production of protease-inhibitor and regulate the trypsin and chymotrypsin hydrolysis activities by more than 90%. Furthermore, Torres et al. (2016) mentioned that heating treatments with boiling or autoclaving could increase the protease activities, following the significant *in vitro* hydrolysis of protease by almost 46%, after exposed to heated legumes. As the protease-inhibitor decreases, the heated winged-bean meal is qualified for further utilization in aquafeed ingredient formulation.

Comparation to nutrient requirements standard in fish

The nutrient requirements for comparative analysis with the unprocessed winged-bean meal, after the heating treatments used several freshwater fish that have many been cultured as the freshwater aquaculture commodities, namely catfish (*Clarias gariepinus*) as carnivorous fish, Nile tilapia (*Oreochromis niloticus*) as omnivorous fish, and Java barb (*Barbonymus gonionotus*) as herbivorous fish. These data are described in Table 2. In Table 2, different food and feeding habit in each fish causes different nutrient requirements. When compared to the nutrient requirements standard provided by Putra et al. (2017) and Rachmawati et al. (2021), the most appropriate winged-bean meal was obtained from the oven-heating process (O, roasting) and non-heating process. This condition emerges as the O and E treatments are mostly included in the nutrient requirements for carnivorous, omnivorous, and herbivorous fish. However, due to trypsin activity regulation in O treatment (Figure 1), the oven-heating treatment is considerable as an alternative plant-based ingredient for aquafeed formulation. Therefore, for fish farmer, it is recommended to formulate aquafeed with the winged-bean meal heated using an oven to reduce the operational cost of feed supply.

Table 2. Comparation data with the nutrient requirements of catfish (C), Nile tilapia (N), and Java barb (J)

Parameter	E (%)	O (%)	A (%)	Nutrient Requirements		
				C (%) ^	N#	J$
Moisture	3.15±0.13c	2.40±0.42b	1.36±0.13a	-	-	-
Ash	4.71±0.67	4.40±0.21	4.45±0.13	-	-	-
Crude Fiber	16.65±4.63	15.30±4.95	12.55±1.85	-	8-10	-
Crude Protein	35.17±0.38c	31.73±1.52b	29.05±1.96a	25-40	30-35	30-32
Crude Fat	10.92±2.95	12.24±4.82	10.14±3.80	9.5-10	10-15	6-7
NFE*	29.41±6.33a	33.93±6.59ab	42.45±2.75b	15-30	40	-

Note: E = unheated winged-bean meal (control), O = oven-heated winged-bean meal, A = autoclave-heated winged-bean meal, *NFE = Nitrogen-free extract, ^, #, $ = standards according to Putra et al. (2017); Rachmawati et al. (2021), respectively. All values are presented as average and standard deviation values. Different superscript letters on the same line show a significant different value (DMRT, p<0.05).

CONCLUSION

Heating treatments, either with oven or autoclave, improve the *in vitro* trypsin activity (1300 unit/O and 1135 unit/A vs 835 unit), but reducing the protein content (29.05±1.96%/A and 31.73±1.52%/O vs 35.17±0.38) and moisture content (1.36±0.13%/A and 2.40±0.42%/O vs 3.15±0.13%). According to the nutrient requirement standard for catfish (25-40%), *Nile tilapia* (30-35%), and *Java barb* (30-32%), the oven-heating treatment and non-heating treatment fulfilled the required standard (27-45%), yet the non-heating treatment showed a decreased *in vitro* trypsin activity value, so the oven-heating treatment is appropriate ingredient for further alternative aquafeed formulation. Nevertheless, the *in vivo* application of winged-bean meal after the heating process should be evaluated further on the digestive activity, nutrient retention, and growth of the target fish to confirm this conclusion.

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