Enhancing Cow Fertility: Leveraging Black Soya, Borlotti, and Jack Beans as Phytoestrogen-Rich Feed Sources

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ABSTRACT

Estrus is a symptom that appears as behavioural receptivity of mating. Under normal circumstances, the estrus cycles in cattle will recover within 30 days after calving. In reality, the calving interval in cattle is more than 85 days. This calving interval is influenced by the imbalance of reproductive hormones. Legumes contain phytoestrogens or estrogen. Estrogenic compounds, including phytoestrogens in small doses, influence the initiation of GnRH from the hypothalamus and accelerate the pituitary gland to release FSH and LH. This study aims to determine the use of Black soya, Borlotti, and Jack beans as phytoestrogen sources in animal feed to decrease the postpartum estrous period in cattle. Our results showed, Black soya and Jack bean with the oven drying method (55^{o}C) and the freezing drying method (-40^{o}C) contain large amount of phytoestrogen used as a source in animal feed to improve fertility. In the administration of this supplement, the animal should be given continuously. Four samples were adhered to a gel silica plate using UV 254 and UV 366 as follows; Black soya dried in the oven (BSFOD), Black soya dried in the freezer (BSFFD), Jack bean dried in the oven (JFOD), and Jack bean dried in the freezer (JFFD). Borlotti bean dried in the oven (BFOD), and Borlotti bean dried in the freezer (JFFD) containing unidentified flavonoids. The results showed only four samples were close to the genistein standard in colour and Rf value (Thin Layer Chromatography Method). The genistein standard has a value of 0.50 and a purple colour, while the BSFOD, BSFFD, JFOD, and JFFD standards have values of 0.48, 0.51, 0.47, as well as 0.49, respectively. The concentration of genistein in BSFOD and BSFFD was significantly different ($P<0.05$), where BSFOD is 0.658g/100g, and BSFFD is 1.784g/100g. Considering these results, Black soya and Jack bean contain large amount of phytoestrogen used as a source in animal feed to improve fertility.

Keywords: Legume's forage, Phytoestrogens, Cow fertility

INTRODUCTION

The Indonesian government has taken a number of steps to meet domestic demand, including enhancing livestock genetic quality and increasing the number and productivity of beef cattle through artificial insemination (AI) technology. The activities to increase the production of beef cattle are limited by the low reproductive performance of these animals, which is caused by a calving interval of 20 months or more, a low birth rate of only 21%, and a calf mortality rate of 18% [1].

By injecting gonadotropin-releasing hormone (GnRH) into post-calving cows with prolonged anestrus, attempts have been made to overcome the first post-calving estrus duration caused by hormonal balance disorders during this time. Officers were unable to get to the farmer's location because of the high cost and limited availability of GnRH, which makes it inappropriate in certain situations. Thus, local ingredients with phytoestrogens that raise GnRH have a lot of potential [2].

The majority of phytoestrogen compounds are isoflavone derivatives found in Glycine soja, Black soya bean seeds, and other leguminous plants like jack beans and Borlotti. According to a prior study, phy-toestrogen functions in the body similarly to estrogen [3]. Additionally, it is a class of naturally occurring steroid hormones that are crucial to the maturation and development of female sexual characteristics [4]. Phytoestrogen then combines with progesterone to halt ovulation, increasing the likelihood of pregnancy developing right away [5].

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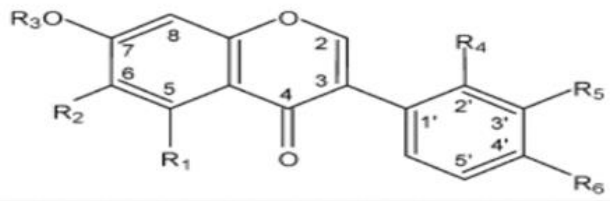
Phytoestrogens are dietary compounds derived from plants that are found in a variety of foods, particularly legumes. Examples of these include Black soya (Glycine max), Borlotti (Lablab purpureus), Jack beans (Canavalia ensiformis), and others [6]. Although genistein has drawn the most attention, the main class of phytoestrogens in black soya beans is isoflavones, which make up more than 65% of the glycosylated form of genistein in products made from black soya beans [2]. Black soya beans are a natural hormone replacement food because their phytoestrogen content satisfies the body's need for estrogen [7, 8].

In addition to Black soya beans, Borlotti is more nutrient-dense than peanuts and walnuts. LC-MS analysis showed that cooked Borlotti bean contain flavonoids, flavones and hydroxycinnamic acids [9]. Walnuts have 127 mg/100 g of phytoestrogen, compared to 104 mg/100 g in peanuts [10, 11].

Furthermore, jack beans can serve as a substitute for Black soya beans in situations where the latter are scarce due to their similar nutritional value [12]. Black soya beans also have a phytoestrogen content that is comparable to that of almonds (183 mg/100 g) and pistachios (276 mg/100 g) [10, 11].

Due to the lengthy sample identification process and the low efficiency of chromatographic separation, genistein analysis in legume samples is complicated [13, 14]. Therefore, HPLC and electrochemistry, UV-vis di-ode-array detector (DAD), and/or detector mass spectrometry (MS) are among the most commonly used methods of identifying isoflavones. For compounds that are subjected to quantitative analysis, the efficient application of chromatography in conjunction with suitable isolation techniques is ideal.

Thus, the purpose of this study is to use various drying techniques to ascertain the isoflavone content in Black soya, Borlotti, and jack beans—a source of phytoestrogens. In Figure 1, the chemical structure is displayed.



Name of isoflavone	R_1	R_2	R_3	R_4	R_5	R_6
daidzin	H	H	glc	H	H	OH
glycitin	H	OCH_3	glc	H	H	OH
genistin	OH	H	glc	H	H	OH
ononin (formononetin-7-O-β-D-glucoside)	H	H	glc	H	H	OCH_3
sissotrin (biochanin A-7-O-β-D-glucoside)	OH	H	glc	H	H	OCH_3
daidzein	H	H	H	H	H	OH
glycitein	H	OCH_3	H	H	H	OH
genistein	OH	H	H	H	H	OH
formononetin	H	H	H	H	H	OCH_3
biochanin A	OH	H	H	H	H	OCH_3

Fig. 1. The chemical formulas for sissotrin, daidzein, genistein, glycitein, formononetin, daidzin, genistin, glycitin, ononin, and biochanin

MATERIALS AND METHODS

Ethical Approval

Ethical approval is not necessary for this study because no live animals were used.

Experimental Plants

The legume plants tested included Black soya beans, Borlotti, and jack beans. Subsequently, planting was conducted in polybags measuring 30 x 30 cm, with 2 to 3 seeds planted in each. In this study, a total of 90 polybags were used as planting media with 3 plants each, which were harvested after 110 days, followed by a drying process, which was performed in 2 cycles, namely, oven drying and freeze dry using low temperatures to identify the process with the highest levels of phytoestrogens.

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Research Material

In this study, the forage of black soya beans, Borlotti, and jack beans, chemicals, silica Gel F254, standard Genistein, and aquades were used. Filter paper, measuring cups, shakers, porcelain cups, ovens, freeze dryers, blenders, 10 ml test tubes, analytical balances with sensitivity of 0.1 mg, electric heaters, water baths, basins, flacons, separating funnels, UV 254 and UV 366 lamps, and a set of densitometry tools were also included in the appliances.

Research Location

This study was conducted for 180 days at Nahdlatul Wathan University Mataram's Experimental Garden, which served as a place for planting legume forage (Black soya beans, Borlotti beans, and jack beans). Meanwhile, the identification of phytoestrogens was conducted at the Testing Service Unit, Faculty of Pharmacy, Airlangga University, Surabaya. At the Laboratorium Nutrisi dan Pakan Ternak, Fakultas Peternakan, Mataram University, the proximate analysis of Black soya bean legume forage, Borlotti as well as a jack. Also, the degradation of dry matter digestibility and organic matter of legumes forage was analyzed at the Laboratorium Nutrisi dan Pakan Ternak, Fakultas Peternakan, Mataram University.

Analysis of Genistein Contain

After being picked straight from the plant, each 100 g forage of black soya, jack, and Borlotti beans was cleaned, drained, and allowed to air dry before being baked at 55 °C to finish drying. In addition, the material was dried at a low temperature (-40 °C) before being ground into a powder.

Five grams of powder were taken out of each legume forage using a vortex, and the powder was filtered through 50 milliliters of ethanol. The resulting filtrate was then evaporated until it solidified. Ten milliliters of hot distilled water and ten milliliters of technical hexane were then added and put in a separating funnel. Following separation, the bottom layer is shaken with 10 milliliters of ethyl acetate.

Each extract's isoflavonoids were determined by measurements made with TLC Densitometry, which allowed for the quantitative detection of genistein in the extract and the subsequent use of a standard genistein curve. On a silica gel plate F254 (20 x 10 cm), 16 µl of each test solution were spotted, with a gap of 1 cm between each spot. Additionally, a chromatographic vessel saturated with the mobile phase toluene-ethyl acetate-formic acid (7 – 3 – 0.1 v/v, upper phase) was used to expand 2 µl of genistein to a height of 7 cm on the same plate as a comparison. Following completion of development, it is detected using UV 254 and UV 366 in comparison to genistein.

The contexture of the isoflavone was determined by reversed-phase HPLC [15]. Meanwhile, the genistein content of each extract was determined by densitometry, which involved quantitative re-spotting of the extract, followed by the standard genistein curve, which was replicated 3 times.

Genistein standard curve creation

A certain amount of genistin was added to TLC silica gel plate F254's 0.5 µl, 1.0 µl, 1.5 µl, and 2.0 µl. Toluene, ethyl acetate, and formic acid (7 – 3 – 0.1 v/v, upper phase) were utilized as the mobile phase to de-velop the solvent. Then, densitometry was used to measure the spots of genistin that had emerged at a wavelength of 270 nm.

Genistein content in leaves or research samples was determined using HPLC analysis [16]. It was identical to the earlier method of manually examining the contents of genistein, though. In order to apply this method, samples are ground up and vortexed for one minute in 2.5 milliliters of a 1:1:1 hexane to methyl tert-butyl ether and methylene chloride extraction solvent. In order to separate the aqueous and organic layers, the samples were also gently vortexed for 15 minutes, followed by a 10-minute centrifugation at 3000 rpm. Each sample's aqueous layer was frozen at 80 °C in the meantime, and the organic layer was transferred into a 10 mL glass conical screw-cap tube and dried at 40 °C using nitrogen gas.

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Mobile phase buffer A (0.05% formic acid and 5 mM ammonium formate in distilled water) was used to re-constitute the dried extracts with the independent controls (genistein, daidzein, glycitein, and their glyco-sides) in a 1:1 ratio of 0.2 mL to mobile phase buffer B (0.05% formic acid and 5 mM ammonium formate in an 80:10:10 ratio, acetonitrile to methanol to distilled water). To get rid of any last bits of insoluble material, the samples were centrifuged for two minutes at 1500 rpm after a vigorous five-minute vortex. Additionally, for every chromatography run, the supernatants were extracted and collected using 0.25 mL polypropylene injection vials with caps. Lastly, a comparison was done between the purity requirements and the areas under curves.

Analysis of Dry Material and Organic Material

The test started with milling oven-dried and freeze-dried Black soya bean forage, then manipulating Tilley & Terry (1963) procedure [17, 18]. First, a total of 0.5 grams of each sample was put in a fermenter tube, and 10 ml of rumen fluid containing 40 ml of McDougall's solution (1:4), irrigated with CO_2, was added. The tube was then immediately closed and incubated in a water bath for 48 hours. After which, 6 ml of 20 % HCL was added per tube gradually to reduce froth. Furthermore, 2 ml of 5 % pepsin was added and incubated for 48 hours, and the contents of the fermenter were filtered with glass wool until it became clear. Next, to calculate the organic matter, the residue was dried in an oven at 105 ^{0}C to determine the residual dry matter, ashed in an electric furnace at 550 ^{0}C. Finally, the digestibility was calculated using the following formula:

$$KCBK\ \ (\%) = \frac{BK\ \ asal - (BK\ \ residu - BK\ \ residu\ \ blanko)}{BK\ \ Asal} x\ \ 10\%$$

$$KCBO\ \ (\%) = \frac{BO\ \ asal - (BO\ \ residu - BO\ \ residu\ \ blanko)}{O\ \ Asal} x\ \ 10\%$$

Description:

KCBK = dry matter digestibility

KCBO = organic matter digestibility

BK = dry material

BO = organic material.

Variables Measurement

The parameters measured were genistein concentration (Rf value), dry material digestibility (DM), and organic material digestibility (OM). Meanwhile, the procedure of Tilley & Terry (1963) was used to determine the content of dry matter and organic matter.

Statistical Analysis

The data used in the identification of phytoestrogen were analyzed descriptively. Moreover, KCBK (dry material digestibility) and KCBO (organic material digestibility) were analyzed using the t-test to determine the difference in the phytoestrogen content of legume forage between the two drying processes [19, 20].

RESULTS

Identification of Phytoestrogens in Legumes Forage

The detection data on UV 254 and UV366 for the 6 samples spotted include BSFOD, BSFFD, BFOD, BFFD, JFOD, JFFD. Based on the results, only 4 have parallel spots and the same colour as genistein, namely light purple. Meanwhile, the other 4 had spots that were not parallel, and the colour was different from genistein. The results of genistein identification with Thin Layer Chromatography (TLC) on samples detected with UV 254 and UV 366 are listed in table 1.

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Table 1. Thin Layer Chromatography (TLC) results on samples detected with UV 254 and UV 366.

No.	Samples	Rf value	Colour
1.	Borlotti bean Forage (BFOD) - oven dryer	0.43	Beige
2	Jack bean Forage (JFOD) - oven dryer	0.47	Violet
3.	Black soya bean Forage (BSFOD) - oven dryer	0.48	Violet
4.	Genistein	0.50	Purple
5.	Black soya bean Forage (BSFFD) - freezer dryer	0.51	Purple
6.	Jack bean Forage (JFFD) - freezer dryer	0.49	Violet
7.	Borlotti bean Forage (BFFD) - freezer dryer	0.44	Beige

The identification results of several types of legumes are shown in Table 2.

Table 2. The identification results of three types of legumes forage

No.	Legumes Forage	Identification
1.	Black soya bean Forage (BSFOD) - oven dryer	+
2.	Black soya bean Forage (BSFFD) - freezer dryer	+
3.	Jack bean Forage (JFOD) - oven dryer	+
4.	Jack bean Forage (JFFD) - freezer dryer	+
5.	Borlotti bean Forage (BFOD) - oven dryer	-
6.	Borlotti bean Forage (BFFD) - freezer dryer	-

Description: + genistein was found / detected

- There was no genistein found

Genistein contains in Black soya bean Forage and Jack bean Forage

The genistein was measured using TLC densitometry, which requires a series of standard solutions since the samples were extrapolated. The genistein contents of oven-dried and freeze-dried Black soya bean and Jack bean forage are presented in Table 3 and Table 4.

Table 3. Genistein Contain of Black soya bean Forage using oven and freezer dryer

Replication	Genistein Contains (g/100g)	
	Black soya bean Forage using oven dryer	Black soya bean Forage using freezer dryer
1	0,675	1,760
2	0,659	1,772
3	0,668	1,763
Average*)	0,667±0,008	1,765±0,006

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Description:*) = significantly different (P<0,05)

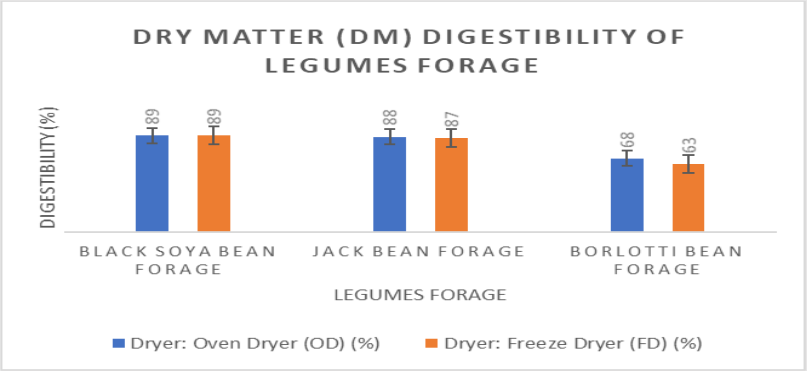
Table 4. Genistein Contain of Jack bean Forage using oven and freezer dryer

Replication	**Genistein Contains (g/100g)**	
	Jack bean Forage using oven dryer	**Jack bean Forage using freezer dryer**
1	0,580	1,665
2	0,564	1,677
3	0,573	1,668
Average*)	0,572±0,005	1,670±0,004

Description:*) = significantly different (P<0,05)

Digestibility of dry material and organic material of legume forage

The digestibility values of dry (DM) and organic material (OM) from legume forage with different drying effects are shown in Tables 5, 6, and 7. The statistical analysis results ('t' test) showed no significant effect of treatment on the digestibility of DM and OM. This implies a difference in the digestibility value of each legume forage, as shown in figure 2.



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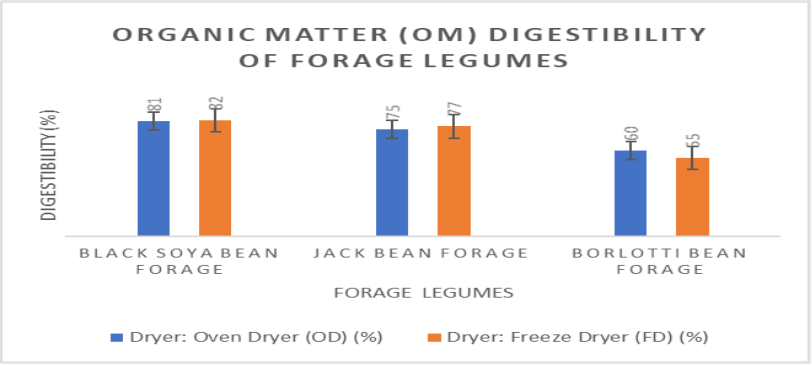

B

Fig. 2. The digestibility values measurement of dry (DM) and organic material (OM) followed the procedure of Tilley & Terry (1963); DM digestibility (A) and OM digestibility (B). Although there was no difference in drying method on DM and OM digestibility, the digestibility value of Black soya bean forage and Jack bean forage was higher than the Borlotti bean forage.

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Table 5. DM and OM digestibility values of Black soya bean forage in two treatments

Feed/Repetition	BK(%)		BO(%)	
	Oven-Dried	Freeze Dried	Oven-Dried	Freeze Dried
Black soya beans Forage				
1	89,428	89,588	83,3045	83,5007
2	88,314	89,004	80,001	81,001
3	88,240	88,480	79,485	80,002
Average^{ns}	88,660±0,67	89,024±0,55	80,930±2,07	81,501±1,80

Description:ns : not significantly affect (P>0,05)

Table 6. Digestibility values of DM and OM from Jack bean forage in two treatments

Feed/Repetition	BK(%)		BO(%)	
	Oven-Dried	Freeze Dried	Oven-Dried	Freeze Dried
Peanut Forage				
1.	86,314	87,627	76,692	77,796
2.	86,218	87,006	75,245	77,204
3.	84,678	85,426	73,565	76,695
Average^{ns}	85,737±0,92	86,686±1,14	75,167±1,56	77,232±0,55

Description:^{ns} : not significantly affect (P>0,05)

Table 7. Digestibility values of DM and OM from Borlotti Bean Forage in two treatments

Feed/Repetition	BK(%)		BO(%)	
	Oven-Dried	Freeze Dried	Oven-Dried	Freeze Dried
Mung Beans Forage				
1.	68,555	63,771	60,263	55,287
2.	65,985	62,857	60,567	54,585
3.	67,885	63,695	60,115	55,008
Average^{ns}	67,475±1,33	63,441±0,51	60,315±0,23	54,960±0,35

Description:^{ns} : not significantly affect (P>0,05)

DISCUSSION

Our results showed that the genistein standard has an Rf value of 0.50 with a purple or light purple colour. The samples of Black soya bean forage which were oven-dried and freeze-dried, have Rf values close to the genistein standard of 0.48 and 0.51. Similarly, the Jack bean forage is close to the standard Rf value of genistein, which is 0.47 at JFOD and 0.49 at JFFD. This showed that the 4 samples contained genistein. However, in the samples of Borlotti bean forage on BFOD and BFFD, the Rf values were not similar or close to the standard, which contained other unidentified flavonoids. This is following a prior study [21] which stated that several factors, such as

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incubation time, cause isoflavones to be undetectable. Isoflavone glycosides cannot be determined in the baso-lateral side without lectin after 4-hour incubation.

Geneistan analysis also affected the identification of Isoflavone content in a material. According to a previous study [22], the first value of isoflavone glycosides was transported to the basolateral side after 4-hour of incubation.

According to studies by [23] , genistein was contained in Black soya beans after Spiral dextrin subfraction (SD-40) was made available through enzyme debranching and gradient ethanol precipitation interacted with Black soya bean isoflavone (SIO) to form V-type inclusion complexes. Meanwhile, the nutritional value of Jack beans is close to Black soya beans. This is evidenced by the Rf value of jack beans approaching the Rf value of Black soya beans, which is in line with the genistein standard.

In this study, the results are also in line with the review [24] , which stated that the highest isoflavone content was found in legumes, especially in Black soya beans. Meanwhile, the highest isoflavone was contained in Black soya beans seeds, especially in the hypocotyl (germ) part that grows into plants. The other part is present in the cotyledons, which become the plant's first leaves. Furthermore, it showed that the roasted, raw, tempeh, tofu, milk, and oil isoflavone content in Black soya beans, is 148.5 mg per 100 gr, 154.53 mg/100 gr, 3.82 mg/100 g, 13.1–34.78 mg /100 g, 5 0.7–10.73 mg /100 g, as well as 0 (does not contain isoflavones).

Besides Black soya beans and their processed products, identifying roots using Thin Layer Chromatography (TLC) showed that it also contained phytoestrogen isoflavone compounds, namely genistein and daedzein. However, the levels of these compounds had not been measured [25,26].

Based on statistical analyses, the genistein between Black soya bean Forage in BSFOD and BSFFD showed a significant effect ($P<0.05$), implying that the drying process significantly affected the genistein contained. Subsequently, Black soya bean forage in BSFFD had higher genistein content of 1.098 g than Black soya bean forage in BSFOD. This was caused by the number of substances that evaporate during the drying process using an oven at a high temperature of 55 ºC, which affected the genistein content in the Black soya bean forage.

During the processing, genistein compounds were transformed through fermentation or non-fermentation, such as heating and others. Therefore, free genistein compounds such as isoflavone are available in different amounts before processing. [16].

The samples of Black soya and Jack beans using a freezer dryer (-40 ºC) produced higher DM and OM digest-ibility values than oven drying (55 ºC). Meanwhile, the legume straw samples of Borlotti beans showed the opposite. The digestibility value by oven drying at 55 ºC was higher than freeze-drying at -40 ºC, in DM and OM, respectively. This follows the information on the nutritional content of some animal feeds [27], which states that the digestibility of DM jack beans is 91.45 % and Black soya bean is 89.41 %. This result is in accordance with [28], stating that the digestibility of DM Black soya bean is 84.19 %.

Tables 5, 6, 7, and Figure 2 shows that the Black soya bean forage sample with oven dryer (55 ºC) generates a DM digestibility of 88.66 %, while freezer dryer (-40 ºC) produces a DM digestibility of 89.02 %. These results are in line with [26], which stated the digestibility of DM in vitro by the oven and freezer dryer for Black soya bean were 58.59% vs. 58.01%, and peanut forage were 58.72% vs. 59.68%, and this indicated it was not affected by the drying method. Meanwhile, the results of [29] showed that the digestibility of DM was influenced by the drying method in *Desmodium rensonii* with 59.74% by oven and 65.62% by freezer dryer.

CONCLUSIONS

Considering these results, it was concluded that Black soya and Jack bean forage contained a large number of phytoestrogens used in animal feed to improve fertility. The dry (DM) and organic material (OM) digestibility values of the 3 legume forages involving Black soya beans, Jack beans, and Borlotti beans, with different drying effects including oven and freezer dryer had no significant impact ($P>0.05$) on the treatment.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data can be provided by the correspondence author.

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