Journal of Sustainable Development; Vol. 17, No. 3; 2024
ISSN 1913-9063 E-ISSN 1913-9071
Published by Canadian Center of Science and Education

Time and Doses of *Bacillus Subtilis* in Leaf Application of Bean Plants

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Received: April 17, 2024 Accepted: May 15, 2024 Online Published: May 20, 2024

doi:10.5539/jsd.v17n3p103 URL: https://doi.org/10.5539/jsd.v17n3p103

Abstract

Common beans *P. vulgaris* is an important food in the diet of the Brazilian population. Currently the use of microorganisms, such as *B. subtillis*, has been increasing significantly as an alternative to improve productivity of beans. The objective of this research was to identify the ideal dose of the commercial product (Panta Premium) based on *B. subtilis*, the best time and doses for leaf application, grain yield and its components and seed quality of bean. The statistical design was randomized blocks arranged in a factorial scheme (3 x 6) with four replicates. Three application times (V3, V4, and R5 stages) and six doses of the inoculant based on *B. subtilis* - strain UFMT Pant001 (0, 200, 400, 600, 800, and 1000 mL per ha^{-1}) were assessed. The number of pods per plant, number of grains per plant, number of grains per pod, 1000-grain weight, grain yield, seed germination, vigor test, first germination count, and accelerated aging test were evaluated. That the 1000-grain weight was not influenced by the different doses and times of *B. subtilis* application. Positive linear regression with an increase in the number of pods per plant as the dose of inoculant (mL ha^{-1}) increased when applied at the R5 stage. V3 and V4 stages, there was a linear increase (43,1 e 28,4% respectively) in the number of grains per plant as the dose of *B. subtilis* increased. At the R5 stage, as seen at the V3 and V4 stages, there is a linear behavior where the number of grains per plant increases as the dose of the inoculant based on *B. subtilis* increases. the results show that both the dose and the time of application of *B. subtilis* showed influence on the physiological quality of the seed. The increasing doses of *B. subtilis* strain UFMT Pant001 increased grain yield compared to the control. The leaf application of *Bacillus subtilis* strain UFMT Pant001 at stage R5 increased the number of pods and grains per plant and grain yield, regardless of the dose. The leaf application of *B. subtilis* strain UFMT Pant001 increased the physiological quality of the seeds, attributed to the good fertility of the soil at the site.

Keywords: *Phaseolus vulgaris* L., productivity, rhizobacteria, microorganisms, growth-promoting bacteria

1. Introduction

In bean *Phaseolus vulgaris* L. (Fabaceae) crop, which is of cultural and socioeconomic relevance in the country, the use of technologies is essential to achieve high yields, among which the use of vigorous seeds that originate high-performance plants associated with inoculation with plant growth-promoting rhizobacteria is characterized as important alternatives for crop production and sustainability (Oliveira et al., 2016).

Coinoculation of multifunctional rhizobacteria can favor an increase in crop productivity, especially greater development of the root system, with greater absorption of water and nutrients and, consequently, greater grain yield (Galindo et al, 2018).

Bacteria from the genus *Bacillus* represent an environmentally friendly strategy for developing biotechnological tools to promote plant growth (Junior et al, 2021). Although its use in reducing the incidence of disease and improving agricultural production is well known, its application is not a widespread practice. The application of Bacillus leaf way means is sustainable for the environment, providing a production system in which the relationship between plants and beneficial organisms is explored and reduces the need for chemical fertilization (Machado et al, 2020).

B. subtilis is considered a plant growth-promoting bacteria (PGPB) due to its interaction with plant roots. Once present, the species has the potential to act in germination and seedling emergence, phosphate solubilization, hormone synthesis, root and shoot growth, biocontrol of plant pathogens and stimulating secondary metabolism (Buchelt et al. 2019).

Foliar application of *B. subtilis* at the R1 stage did not influence plant height, grain yield, and its components of irrigated beans (Silva et al., 2017). Microorganisms of the *Bacillus* group associated with corn plants bring several advantages, as they produce antibiotics, phytohormones, and enzymes, promoting plant growth and biological control of phytopathogens (Buchelt et al., 2019). The application of *Bacillus subtilis* in second-crop corn plants, with inoculation of the seeds, promoted more growth of the shoot of the plants by approximately 15% (Mazzuchelli et al., 2014).

In this context, the present study aimed to identify the ideal dose of the commercial product based *Bacillus subtilis* to be recommended, the best time for leaf application, grain yield and its components, and seed quality of bean.

2. Material and Methods

The experiment was conducted in the field during the fall/winter period of 2022, between May and August, and in the multidisciplinary laboratory, both located at the Universidade Estadual de Goiás, Câmpus Sul, Unidade Universitária de Ipameri, county of Ipameri-GO.

The TAA DAMA cultivar was used, which has the following characteristics: the life cycle ranges from 85 to 95 days; prostrate; the height is 50 cm; the growth habit is indeterminate type (III); the shape of the grain is oblong; the color of the grains is light beige with light streaks; the 1000-grain weight is 280 g; the final population ranges between 180 and 200 thousand plants. It is tolerant to anthracnose, susceptible to bean rust, moderately resistant to angular leaf spot and bacterial disease complex, moderately susceptible to powdery mildew, and resistant to common mosaic.

The experiment was conducted under field conditions at the experimental farm of the State University of Goiás in Ipameri, at 17º 46' 30.3" S, 48º 19' 15.6" W, and an altitude of approximately 800 m. Ipameri has a climate classified as Aw-type, tropical, with a dry season in winter (Cardoso et al., 2014). The temperature is 25º C, with average relative humidity ranging from 58 to 81% and average annual rainfall of 1,447 mm, with around 80% of the rain concentrated in December, January, and March, while the rest in October, November, and February. The soil in the experimental area was classified as Oxisols (United States, 2014) and as a dystrophic Red-Yellow Oxisol (EMBRAPA, 2018). The soil chemical and particle-size attributes were assessed before the experiment was set up, and the results are shown in Table 1.

Table 1. Chemical and physical attributes of the soil, at 0-20 cm layer, before the experiment was set up

P (Melich)	OM	pH	K	Ca	Mg	H+Al	Al	CEC	BS
mg dm^{-3}	g dm^{-3}	$CaCl_2$			cmol$_c$ dm^{-3}				%
11.6	18	5.1	0.26	2.2	0.6	2.5	0	5.66	54.06
Clay (g kg^{-1})	Sand (g kg^{-1})	Silt (g kg^{-1})							
370	540	90							

OM - organic matter; CEC - cation exchange capacity; BS- base saturation

The planter was adjusted to obtain 11 seeds per meter, resulting in a stand of 10 plants per meter, consequently a population of 200 thousand plants per hectare. Fertilization at planting was conducted according to crop needs and soil analysis recommendations, using the NPK formulation 05-10-10 at a dose of 600 kg per ha^{-1} (3 kg 05-10-10 per plant). The soil was prepared with plowing and harrowing to incorporate the crop remains. The beans were sown on May 27, 2022, in the winter crop season or third crop season, with sprinkler irrigation, using NY-30 model sprinklers with a working pressure of 30 mwc, a flow rate of 2.66 m^3 ha^{-1}, and installed 1.5 m from the ground, with a spacing of 12 x 12 m.

During the experimental period it was not observed any disease, but three applications of insecticide were necessary at a dose of 250 g per L^{-1} of Imidacloprid and 50 g per L^{-1} of Bifenthrin, distributed in two applications at the V2 stage and one at the V3 stage, at dose of 250 mL per ha^{-1} to control whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) and cucurbit beetle *Diabrotica speciosa* Germar (Coleoptera: Chrysomelidae). Weeds were controlled by manual weeding in June and July. Harvesting (R9 stage) was conducted manually from September 15 to 19, 2022.

The statistical design was randomized blocks arranged in a factorial scheme (3 x 6) with four replicates. Three application times (V3, V4, and R5 stages) and five doses of the inoculant based on *Bacillus subtilis* - strain UFMT Pant001 (0, 200, 400, 600, 800, and 1000 mL ha^{-1}) were evaluated. Each experimental unit consisted of five rows 5 m long with a spacing of 0.45 m between rows.

Applications were conducted via leaf spray using a manual knapsack sprayer, with a spray volume equivalent to 200 L per ha^{-1} (1 L per plant).

Evaluations were conducted at the end of the crop cycle, at the R9 phenological stage, and the grain yield (13% moisture) and yield components (number of pods per plant, number of grains per plant, number of grains per pod, and 1000-grain weight) were analyzed. For the yield components, ten plants were also collected from each plot and taken to the laboratory to determine:

Number of pods per plant: obtained from the ratio between the total number of pods and the total number of plants; number of grains per plant: obtained from the ratio between the total number of grains and the total number of plants; average number of grains per pod: obtained from the ratio between the total number of seeds and the total number of pods;1000-grain weight: eight sub-samples of 100 grains were used, weighed on an analytical balance (0.1t g), according to BRASIL (2009), and the average values were expressed and grain yield was determined based on the production of the useful area of the plot (three central rows of 5 meters), with moisture of 13%.

After evaluating the grain yield and its components, the physiological attributes of the grains were also evaluated. To germination and vigor test, four replicates of 50 seeds were used in each treatment on paper (Germitest®) rolls moistened with a quantity of distilled water equivalent to 2.5 times the mass of the paper. The paper rolls were placed in plastic bags, sealed, taken to the BOD (Biological organism development) incubator, and kept at 20 °C. Evaluations were conducted on the 15º day after the test was set up. The results are expressed as a percentage of normal seedlings (BRASIL, 2009).

The first germination count was conducted at the same time as the germination test described previously. The percentage of germinated seedlings was observed on the 15º day after the test was set up (BRASIL, 2009).

To accelerated aging test, acrylic plastic boxes containing 40 mL of water and aluminum mesh were used. Four replications of 50 seeds for each treatment were distributed in a single uniform layer on the aluminum mesh in the boxes. The boxes were kept in a BOD chamber at 42 °C for 72 hours. After the aging period, the seeds were subjected to the germination test (GER), following the standards established by BRASIL (2009).

The data obtained were subjected to analysis of variance, and for the qualitative factor (seasons) the Tukey test was carried out at 5% probability and for the quantitative factor (doses) the polynomial regression study was carried out. To carry out statistical analyzes the SISVAR program was used (Ferreira, 2011).

3. Results and Discussions

The results of grain yield, yield components, and physiological attributes of grains are shown in Tables 2 and 3, respectively. In Table 2, the F-test evidences that the 1000-grain weight was not influenced by the different doses and times of *B. subtilis* application. The beans were grown in an experimental area with a high soil fertility (Table 1) due to residues from previous crops, which may have contributed to the results presented in this study. However, the interaction between doses and times of application of *B. subtilis* influenced the number of pods per plant, number of grains per plant, and grain yield. The number of seeds per pod was affected only by the doses of *B. subtilis* (Table 2).

Table 2. Number of pods per plant (NPP), number of grains per plant (NGP), number of grains per pod (NGPD), 1000-grain weight (1000W), and grain yield (GY) according to the doses of an inoculant based on *Bacillus subtilis* - strain UFMT Pant001 applied at different times to the bean crop. UEG, 2022, Ipameri-GO.

Source of variation	Degrees of freedom	Mean squares				
		NPP	NGP	NGPD	1000W	GY
Dose	5	4.884**	10.896**	3.183*	0.681^{ns}	901.322**
Application time	2	1.528^{ns}	7.345**	0.827^{ns}	0.011^{ns}	343.637**
Block	3	0.709^{ns}	2.643^{ns}	3.189^{ns}	1.121^{ns}	0.677^{ns}
Dose * Application time	10	2.284*	5.033**	0.654^{ns}	0.743^{ns}	3.835**
CV(%)		24.45	15.32	9.77	6.22	2.99

CV (%) - Coefficient of variation; * - Significant at $p \leq 0.05$ by the F test; ** - Significant at $p \leq 0.01$ by the F test; ns - Not significant.

Figure 1A shows that the results fit into a positive linear regression with an increase in the number of pods per plant as the dose of inoculant (mL ha^{-1}) increased when applied at the R5 stage, as was also observed in Figure 1B for the number of grains per plant. These two variables are directly linked to crop yield; consequently, there is an increase in grain yield according to the dose of the product applied (Figure 1 D).

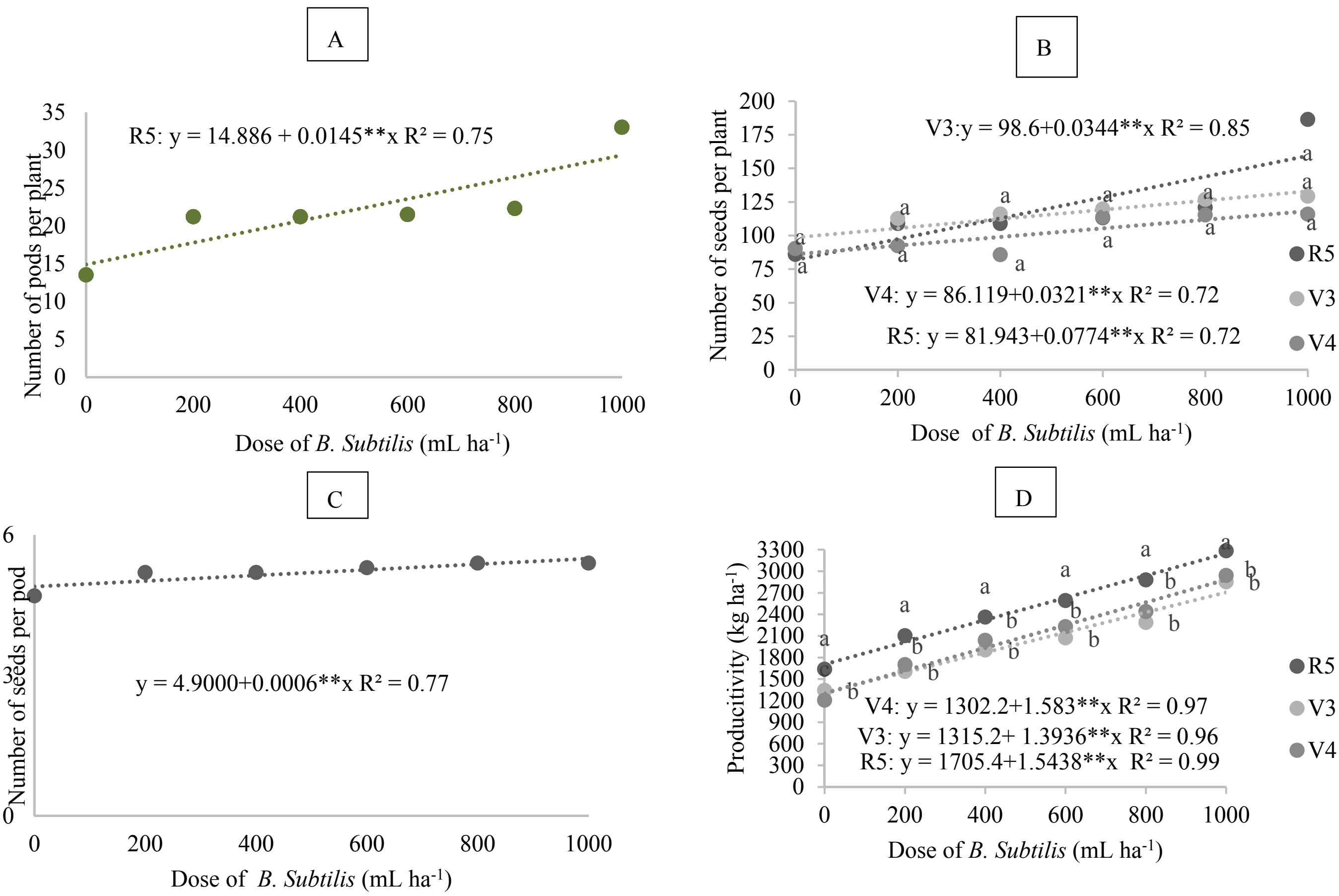

Figure 1. Number of pods per plant in R5 application stage (A), number of seeds per plant (B), number of seeds per pod (C), productivity (D) in the irrigated bean crop in function of doses of *B. subtilis* applied in the leaves. UEG, 2022, Ipameri-GO

According to Queiroz et al. (2023) this increase may be related to some mechanisms of action of this bacterium, such as the production of growth-promoting substances and improved plant nutrition. *B. subtilis* is a soil bacterium that produces hormones such as indole-acetic acid (IAA) and indole-butyric acid (IBA) and secretes enzymes that are important for plant nutrition, as shown by the results presented by Araújo (2008). *Bacillus* species can form endospores that are extremely resilient to adverse environmental conditions and can also secrete metabolites that stimulate plant growth and vigor. Thus, successfully applying beneficial bacteria increases stress tolerance and adaptation to climate change (Hashem et al., 2019).

Figure 1B shows that at the V3 and V4 stages, there was a linear increase (43,1 e 28,4% respectively) in the number of grains per plant as the dose of *B. subtilis* increased. This can be attributed to the fact that the inoculant improved the plant's development in the vegetative phase, which is the most susceptible to diseases, acting on the plant's defense mechanisms and also boosting nitrogen absorption since this stage, which precedes flowering, is the phase in which the bean plant most needs this nutrient, for the formation of pods and grains (Silva et al., 2017).

At the R5 stage, as seen at the V3 and V4 stages, there is a linear behavior where the number of grains per plant increases as the dose of the inoculant based on *B. subtilis* increases. This is relevant because the number of pods per plant is an important crop yield component. Therefore, by applying a product that can increase this component, there is the possibility to increase yield.

The number of grains per plant increased according to the increase in the inoculant doses (Figure 1B); the application at the R5 stage provided the best result. This can be explained by the fact that during this stage, the plant rapidly accumulates nutrients, which must be balanced to ensure the production of flowers; also, the biological nitrogen fixation (BNF) activity declines, so there is greater absorption by the plant, increasing the number of pods.

Figure 1C shows a linear graph showing that the higher the dose of B. subtilis, the higher the number of grains per pod, regardless of the stage at which it is applied. The bacterium acts by improving the absorption of water and nutrients and the production of antibiotics, phytohormones, and enzymes, providing growth promotion and biological control of phytopathogens, giving the plant greater resistance, and increasing the number of grains per pod (Melo et al., 2021). A different result was observed by Zucareli et al. (2018) when they found that the application of Itafós (Brazilian phosphate Itafós has low reactivity, in addition to being less concentrated, with 23.2% total P2O5 and 5.5% P2O5 soluble in 2% citric acid) associated with inoculation with B. subtilis did not influence the number of grains per pod in the bean crop.This result can be explained by the effectiveness of B. subtilis in strengthening the plant defenses, improving nutrient absorption and growth as a result of increased nitrogen fixation and phytohormone synthesis, triggering an increase in metabolites that sensitize the root system to the external conditions of the environment, even providing greater plant capacity to absorb nutrients (Melo et al., 2021). Corroborating the report by Abhilash et al. (2016), multifunctional microorganisms, when associated with the plant, can promote its growth and a series of other benefits through different mechanisms of action, being expressed at various stages of plant development and presenting positive results with a direct impact on yield as was observed in this study (Figure 1C).

There was an influence of the interaction between doses and times of application of inoculant based on *B. subtilis* on the grain yield (Figure 1C). As the doses of inoculant based on *B. subtilis* increased, there was an increase in bean yield, and the best results were observed when the application was conducted at the R5 stage. All yield components showed significant results, consequently increasing bean grain yield (Figure 1D). Zucareli et al. (2018) obtained similar results to this study in bean crop.

According to Junior et al. (2018) the inoculation of this bacteria promotes greater grain yield and crop protection in the field, corroborating the results of this study. However, divergent results were obtained by Silva et al. (2017) where inoculation with *B. subtilis* via aerial part, associated or not with fertilization foliar with nitrogen and boron, not influenced the components of grain production and productivity in the irrigated bean plant, as the application of the bacteria via aerial part can generate a microclimate stressful for growth and bacterial development, reducing its benefits to the culture of interest.

The doses and times of application of inoculant based on *B. subtilis* influence the physiological quality of the seeds (Table 3).

Table 3. First germination count (FC), germination (GM), and accelerated aging (AG) of seeds of the irrigated bean crop according to the doses of inoculant based on *B. subtilis* supplied via leaf application at the V3, V4, and R5 stages. UEG, 2022, Ipameri-GO

		Mean squares		
Source of variation	Degrees of freedom	FC	GM	AG
Dose	5	8888889**	8888889**	3066667**
Application time	2	14222222**	14222222**	0.666667**
Block	3	0.00000000E+0000**	0.00000000E+0000**	0.00000000E+0000**
Dose * Application time	10	3555556**	3555556**	6533333**
CV(%)		0	0	0

CV- coefficient of variation; ns - not significant

In Table 3, the results show that both the dose and the time of application of *B. subtilis* showed influence on the physiological quality of the seed. One of the factors that justifies this result is that the experiment was conducted in a single harvest and it is known that multifunctional microorganisms provide essential nutrients to crops in the soil solution (Arruda, 2012). The average value of 98.3%, is above the standard required for the production and marketing of bean seeds, since the minimum percentage must be 70% for basic seeds and 80% for certified (C1 and C2) or non-certified (S1 and S2) first and second generation seeds (BRASIL, 2009).

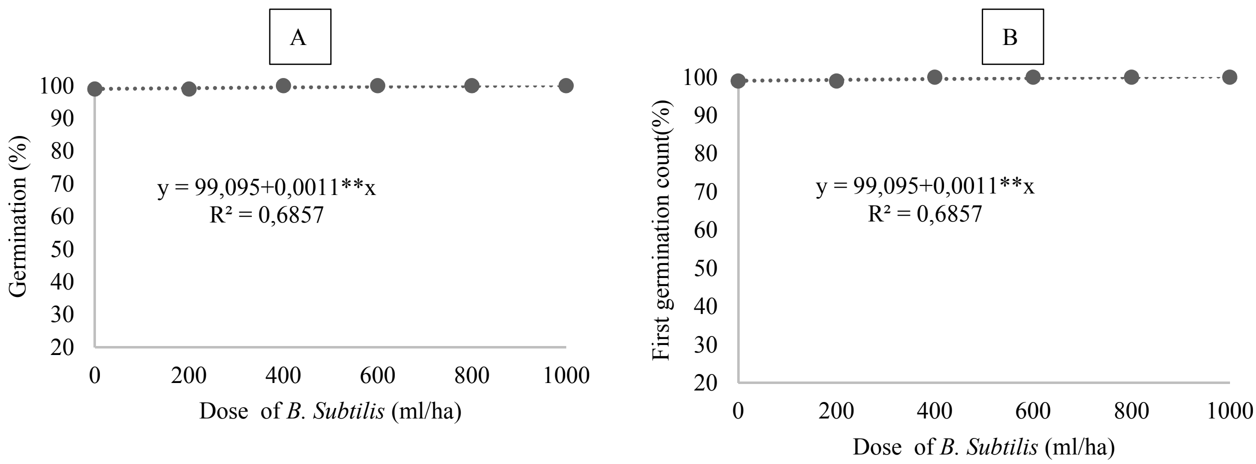

Figura 2. First germination count (A) and germination of seeds (B) of the irrigated bean crop according to the doses of inoculant based on B. subtilis supplied via leaf application at the V3 stages (V4 and R5 had no significant effect). UEG, 2022, Ipameri-GO

Leão et al. (2016) obtained different results and observed that treatments with *B. subtilis* promoted a higher germination percentage in bean seeds under controlled conditions (*in vitro*). As previously reported, under field conditions, rhizobacteria are subject to different adversities, such as interaction with other microorganisms, soil type, moisture, pH, and temperature, which prevent them from expressing their maximum potential as they do under controlled conditions. According to Silva et al. (2017), the application via the leaf of *B. subtilis* can generate a stressful microclimate for bacterial growth and development, reducing its benefits for the crop of interest.

4. Conclusion

Increasing the dose of inoculant based on *B. subtilis* strain UFMT Pant001 resulted in higher grain productivity (100% stage R5, 112.2% stage V3 and 143.7% stage V4). Foliar application of *Bacillus subtilis* strain UFMT Pant001 at the R5 stage led to higher increases in the number of grains per plant and yield, regardless of the dose applied. Foliar application of *Bacillus subtilis* strain UFMT Pant001 improve the physiological quality of the bean seeds.

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Acknowledgment

“Financial resources from BIONSUMOS Notice/Call n. 32/2022, Term of Promotion nº. 000036400598 SEI process nº. 202200020023139”. The authors thank the Universidade Estadual de Goias for the financial support provided throught Call Notice nº 21/2022- Pro-Programs. The Coordination for the Improvement of Higher Education Personnel (CAPES) provided a scholarship for the first author during their master’s degree.

Authors contributions

Prof. Dr. Mariana Pina da Silva Berti and Eliane Aparecida Silveira Ferreira were responsible for study design and revising. Eliane Aparecida Silveira Ferreira and Claudiene Cristina da Silva was responsible for data collection. Prof. Dr. Mariana Pina da Silva Berti and Eliane Aparecida Silveira Ferreira drafted the manuscript and Dr. Mariana Pina da Silva Berti and Dr. Fabricio Rodrigues revised it. All authors read and approved the final manuscript.

Funding

This study was financed Financial resources from BIONSUMOS Notice/Call n. 32/2022, Term of Promotion nº. 000036400598 SEI process nº. 202200020023139” and “Call Notice nº 21/2022- Pro-Programs”

Competing interests

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

Informed consent

Obtained.

Ethics approval

The Publication Ethics Committee of the Canadian Center of Science and Education. The journal’s policies adhere to the Core Practices established by the Committee on Publication Ethics (COPE).

Provenance and peer review

Not commissioned; externally double-blind peer reviewed.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Data sharing statement

No additional data are available.