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INVESTIGATION OF THE VARIATION OF GYMNEMAGENIN CONTENT IN *GYMNEMA* R.BR UNDER THE INFLUENCE OF CERTAIN STRAINS OF BACTERIA

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Summary

The capacity of *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, and *Bacillus subtilis* to increase the content of gymnemagenin in *Gymnema* spp. R.Br was investigated on two samples of *G. sylvestre* and two samples of *G. latifolium* by incubating these samples for 8 days with each strain of bacteria. The difference in content of gymnemagenin before and after incubation was determined by using a validated HPLC method meeting all requirements of AOAC International on analytical method performance. The four strains of bacteria were found to increase the content of gymnemagenin in the tested samples after incubation, but the change caused by *B.subtilis* was not statistically significant. No significant strain-to-strain difference in the capacity to improve the content of gymnemagenin was found among the four strains of bacteria.

Keywords: *Gymnemagenin; Biotransformation; Incubation.*

1. Introduction

Gymnemagenin is an aglycone existing either in free form in *Gymnema* R.Br or in the form of gymnemic acids, which can be hydrolyzed chemically or enzymatically to release gymnemagenin (Fig. 1) [1].

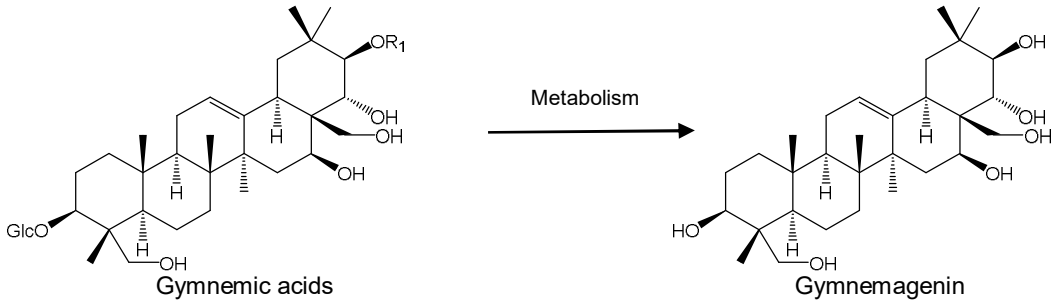

Fig. 1. Relationship between gymnemic acids and gymnemagenin

Gymnemagenin is able to reduce the glucose level in blood [2], possessing anti-obesity and anti-diabetes capacities [3], and it is considered to have important therapeutic potential.

During their growth phase, microorganisms produce enzymes capable of degrading natural compounds in plants to transform them into new entities [4]. The enzymatic transformation of natural products, such as that of gymnemic acids, can be catalyzed by different types of bacterial enzymes, such as glucosidases secreted by *L. casei* [5], *L. rhamnosus* [6], *B. subtilis* [7], or *B. bifidum* [8], etc. Among other cases, gymnemic acids in extracts from *G. sylvestre* were found to be hydrolyzed to release gymnemagenin after incubation with *B. bifidum*, *L. casei*, and *L.rhamnosus* [9]. Therefore, bacterial fermentation is a promising approach to transform gymnemic acids into free gymnemagenin to increase the content of gymnemagenin in *Gymnema* R.Br's raw materials.

This study was carried out to investigate the capacity of four bacterial strains (*B. bifidum*, *B. subtilis*, *L. casei*, and *L. rhamnosus*) to increase the content of gymnemagenin in *Gymnema* R.Br by enzymatic biotransformation of gymnemic acids, with focus on the impact of certain parameters of the fermentation process (incubation time, the ratio between the mass of plant material and the bacterial suspension during the incubation process, and the choice of bacterial strain) on the effectiveness of increasing the content of gymnemagenin.

This effectiveness was quantitatively assessed by monitoring the content of gymnemagenin in

Gymnema R.Br samples before and after incubation with an HPLC method developed and validated according to current requirements of AOAC International [10].

2. Materials and methods

2.1. Materials

2.1.1. Plant materials:

In this study, four samples of *Gymnema* R.Br, including two samples of *G.sylvestre* (coded as G5/2/2/2a, and G5/0) and two samples of *G. latifolium* (coded as G4/5 and G4/11), were used to investigate the capacity of bacteria to increase their content of free gymnemagenin. All samples (consisting of leaves and young stems) were harvested at Yen Ninh ward, Phu Luong district, Thai Nguyen province (currently Yen Trach ward, Thai Nguyen province) in November 2024. After being harvested, the samples were finely cut and dried at 55^{o}C until their humidity did not exceed 8.5% and stored as such until they were used. The content of gymnemagenin in samples was determined by the HPLC method as described in section 2.2.2 before incubation.

2.1.2. Strain of bacteria:

Bifidobacterium bifidum (10^{10} CFU/g), *Bacillus subtilis* (3×10^{10} CFU/g), *Lactobacillus casei* (10^{10} CFU/g), and *Lactobacillus rhamnosus* (10^{10} CFU/g) were used to investigate their capacity to increase the content of gymnemagenin in *Gymnema* R.Br. These strains of bacteria were provided by the Laboratory of Pharmacology and Biochemistry, National Institute of Medicinal Materials, and were used as such to prepare bacteria suspension following the procedure described in section 2.2.1.

2.1.3. Chemicals, reagents, and reference substances:

Reference substance of gymnemagenin (purity: 98.1%, CAS: 22467-07-8, batch no: BCCM 9489) was purchased from Sigma Aldrich (St. Louis, MO, USA).

HPLC-grade solvents (acetonitrile, methanol), PA-grade solvents (acetone, butanol, ethyl acetate, ethanol), and chemicals (potassium hydroxide, hydrochloric acid) were purchased from Merck Vietnam (Ho Chi Minh City, Vietnam). Tryptic soy agar and Tryptic soy broth were purchased from Hemidia (India) and prepared according to the Vietnamese Pharmacopoeia, 5th Edition. Distilled water was employed in this study, with proper additional treatment wherever necessary to meet the corresponding requirements of specific works.

2.1.4. Instruments and equipment:

- Sterilizing processes were done by using the autoclave HV- 110 of Hirayama (Tokyo, Japan).

All HPLC analysis were carried out using an HPLC – DAD system of Thermo Fischer Scientific (Waltham, MA, USA) on a Luna® C18 column (250 mm × 4.6 mm, 5μm) of Phenomenex (Torrance, CA, USA). The glassware and other instruments were of proper standards for general laboratory use, as well as meeting the particular requirements of each specific work**.**

2.2. Methods

2.2.1. Incubation of plant samples with bacteria:

For each *sample preparation*, about 0.75 g of plant sample, previously finely ground, homogenized, and the content of gymnemagenin was determined by HPLC analysis, was accurately weighed into a suitable flask and wetted by mixing with sufficient water, then sterilized in an autoclave at 121^{o}C for 15 minutes and cooled down to room temperature before use.

Bacteria stock suspensions were prepared separately for each strain of bacteria by dispersing about 1.0 g, accurately weighed, of the strain in 20 mL of sterile Tryptic soy broth and incubating at 35^{o}C ± 2^{o}C for 24 hours. Ten milliliters of each stock suspension were transferred into separate flasks, each containing 50 mL of sterile Tryptic soy broth and incubated at 35^{o}C ± 2^{o}C for 48 hours. The formed colony-forming units (CFU) were counted and the incubation process would be continued if necessary until suspensions containing about 10^{8} CFU/mL. These suspensions were used as *bacteria suspension*.

In preliminary studies, in order to estimate the optimal ratio between the amount of plant sample and the volume of bacteria suspension, incubation time was fixed at 10 days and the volumes of *bacteria suspension* were tested at 20, 50, 70, and 100 mL for each *sample preparation*; in order to find out the optimal incubation time for assessing the impact of bacteria on the content of gymnemagenin, the incubation times were tested at 2 days, 5 days, 8 days, and 10 days and one *sample preparation* was incubated with 70 mL of *bacteria suspension*. A summary of these preliminary studies was provided in section 3.2.

From the results of preliminary studies, to assess the capacity of bacteria to increase the content of gymnemagenin in samples, different portions of *sample preparation* were incubated separately with 70 mL of *bacteria suspension* of each strain at 350°C ± 2°C for 8 days. After incubation, the content of gymnemagenin was determined by HPLC analysis.

2.2.2. Determination of gymnemagenin content:

2.2.2.1. Sample preparation:

To determine the gymnemagenin in samples before incubation, about 0.75 g of a plant sample, accurately weighed, was transferred into a flask, then 22.5 mL of ethanol 50%, 2 mL of 12% solution of potassium hydroxide were added into the flask. The flask was connected with a reflux condenser and heated in a water bath set at 100°C for 1 hour, then it was cooled back to room temperature. About 5.5 mL of 4 N solution of hydrochloric acid was added into the flask, and it was heated again in a water bath set at 100°C for 2 hours and cooled back to room temperature. The pH of the liquid inside the flask was adjusted to 7.5 – 8.5 with 12% solution of potassium hydroxide, then the liquid was transferred to a 100-mL volumetric flask, and ethanol 50% was added to the volume. The obtained liquid was filtered through a 0.45 μm filter membrane and injected into the HPLC system.

To determine the gymnemagenin in the sample after incubation with bacteria suspension, the sample was evaporated to dryness and prepared similarly to the sample before incubation.

Stock standard solution of gymnemagenin was prepared by dissolving about 30 mg reference substance of gymnemagenin, accurately weighed, in a 20-mL volumetric flask with methanol and diluting with methanol to volume. Working standard solutions were prepared by diluting the stock standard solution with ethanol 50% to targeted concentrations from 0.03 mg/mL to 0.3 mg/mL.

For accuracy validation of the HPLC method by recovery studies, spiked solutions were prepared by adding an accurate quantity of standard gymnemagenin into the sample preparation at 3 different levels (corresponding to spiked concentrations of gymnemagenin in analyzed solutions of about 0.03 mg/mL, 0.06 mg/mL, and 0.12 mg/mL). For precision validation, six different portions of a sample were prepared and analyzed by the same analyst in the same day for assessment of repeatability and by each one of two different analysts for assessment of intermediate precision. For estimation of limit of quantitation (LOQ) and limit of detection (LOD) of gymnemagenin, spiked samples used for recovery studies were accurately diluted and analyzed to estimate the signal-to-noise (S/N) ratio for the gymnemagenin peak. The concentrations of gymnemagenin at LOD and LOQ corresponded to values of S/N about 3 and 10, respectively. The solvent solution for specificity validation was prepared similarly to sample preparation, without using a plant sample.

2.2.2.2. Chromatographic analysis:

Chromatographic determination of gymnemagenin in samples was carried out on a Luna® C18 column (250 mm × 4.6 mm, 5 μm) employing a mixture of acetonitrile – phosphate buffer maintained at a flow rate of 1.6 milliliter per minute as mobile phase. The composition of the mobile phase varied according to a gradient program: 0 – 20 minutes: 25 – 55% acetonitrile; 20 – 25 minutes: 55 - 60% acetonitrile; 25 – 30 minutes: 60% acetonitrile; 30 – 35 minutes: 60 – 25% acetonitrile; 35 – 40 minutes: 25% acetonitrile. The chromatograms were recorded at 210 nm using UV detection after injecting 50 μL of sample into the HPLC system. The phosphate buffer was prepared by dissolving 0.14 g of potassium dihydrogen phosphate and 0.5 mL of phosphoric acid in 900 mL of water, then water was added to make 1000 mL.

The HPLC method was validated according to current requirements on performance of analytical methods recommended by AOAC International [6]. A summary of validation results was provided in section 3.1.

2.2.3. Assessing the capacity of bacteria to increase the content of gymnemagenin:

To evaluate the capacity of bacteria to increase the content of gymnemagenin in *Gymnema* R.Br's samples, the gymnemagenin content of each sample was determined before and after incubation to detect any change that occurred during the incubation with bacteria suspensions. The statistical significance of the change was assessed by a paired T-test.

2.3. Data processing

Chromatographic data were collected and processed by Chromeleon software of Thermo Fischer Scientific (Waltham, MA, USA). Statistical calculations were done using Microsoft Excel software (Redmond, WA, USA).

3. Results and discussion

3.1. Validation of the HPLC method for the determination of gymnemagenin

The HPLC method gave a well-shaped peak of gymnemagenin, completely separated from other peaks in the chromatogram of sample preparation (Fig. 2, B) with the same retention time and with a similar UV spectrum (Fig. 2, D) to that of the peak of gymnemagenin in the chromatogram of standard solution (Fig. 2, A). In the chromatogram of the solvent solution (Fig. 2, C), no peak was detected at the retention time of gymnemagenin. Therefore, the method was sufficiently specific for the determination of gymnemagenin.

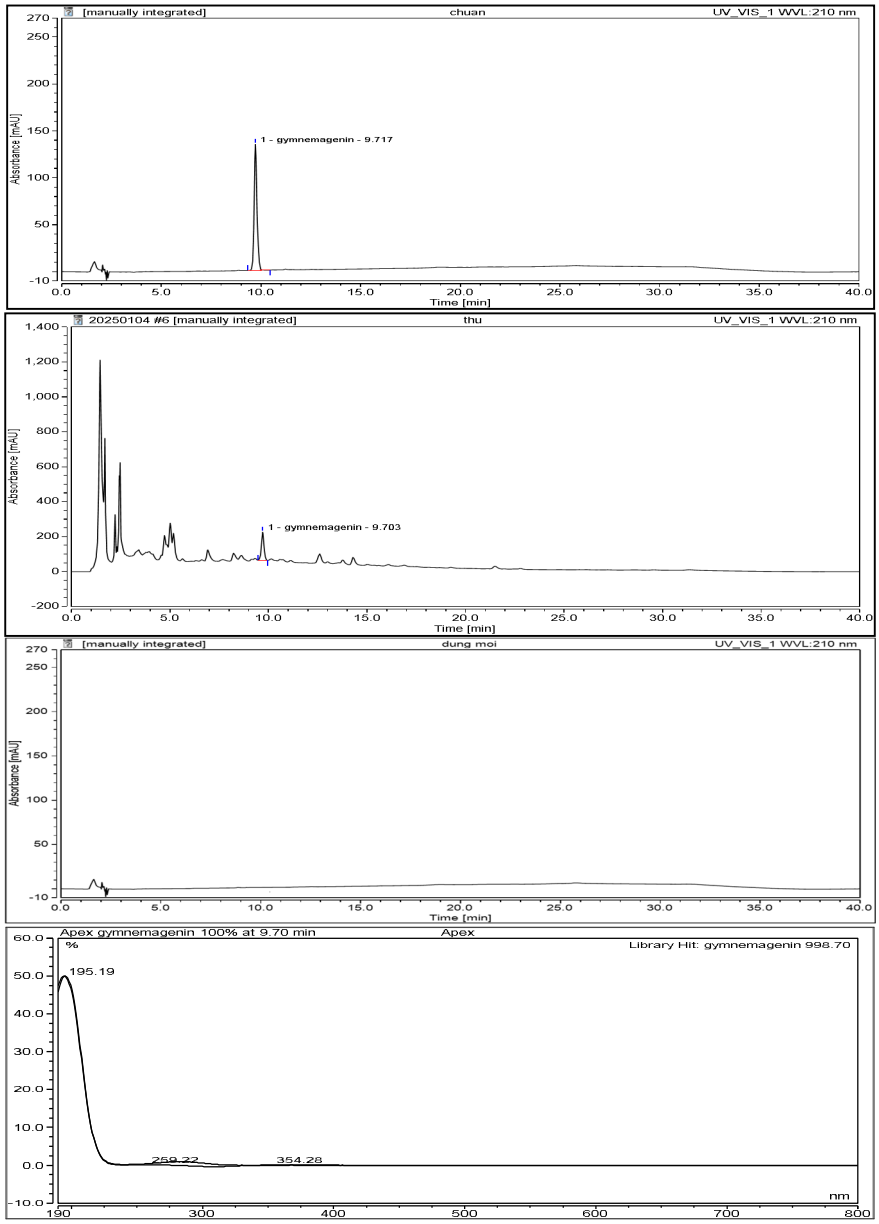

Fig. 2. Typical results for specificity validation (A: chromatogram of standard solution, B: chromatogram of sample preparation, C: chromatogram of solvent solution, D: overlaid UV-Vis spectra extracted from gymnemagenin's peak on chromatograms of standard solution and sample preparation).

The suitability of the chromatographic system was assessed by six consecutive injections of the same standard solution. After six analyses, the RSD (%) values for retention time and area of gymnemagenin peak were 0.02% and 0.08%, respectively, and the gymnemagenin peak was symmetric (with asymmetric factor about 1.2) and the chromatographic system was efficient (number of theoretical plates higher than 2000 for peak of gymnemagenin), confirming the suitability of the chromatographic system for determination of gymnemagenin.

The validation results of accuracy, precision, and estimation of LOD and LOQ were summarized in Table 1. The accuracy and precision of the method were in line with the requirements of AOAC International [6]. The linearity was maintained between concentration and peak area of gymnemagenin within concentration range from 0.03 to 0.3 mg/mL (corresponding to gymnemagenin content in plant samples from 0.4% to 4%), and the LOQ for gymnemagenin content in plant samples was estimated at 1.4×10^{-2} % (w/w).

Table 1. Summary of validation results for HPLC method

Criteria	Requirements	Results
Linearity	$R^2 \geq 0.999$	Concentration range: 0.03 – 0.3 mg/mL $R^2 = 0.9999$
Accuracy		
Level 1 (0.03 mg/mL or 0.4% (w/w) in plant samples, n = 3)	Recovery rates: 95 – 105 % RSD ≤ 3.7 %	Recovery rates: 98.7 – 98.9 % RSD = 0.1 %
Level 2 (0.06 mg/mL or 0.8% (w/w) in plant samples, n = 3)	Recovery rates: 95 – 105 % RSD ≤ 3.7 %	Recovery rates: 98.8 – 98.9 % RSD = 0.1 %
Level 3 (0.12 mg/mL or 1.6% (w/w) in plant samples, n = 3)	Recovery rates: 97 – 103 % RSD ≤ 2.7 %	Recovery rates: 99.2 – 101.4 % RSD = 1.2 %
Precision		
Repeatability (n = 6, 1 analyst), gymnemagenin content in the sample was about 2% (w/w)	RSD ≤ 2.7 %	RSD = 0.8 %
Intermediate precision (2 analysts, n = 6 for each analyst), gymnemagenin content in the sample was about 2% (w/w)	RSD ≤ 2.7 %	RSD = 2.2 %
LOQ		1.1 µg/mL (equivalent to gymnemagenin content about $1.4*10^{-2}$ % (w/w) in plant samples)
LOD		0.6 µg/mL (equivalent to gymnemagenin content about $0.8*10^{-2}$ % (w/w) in plant samples)

3.2. Determination of some parameters for the incubation process

Preliminary studies were carried out to find a suitable ratio between the amount of plant sample and the volume of bacteria suspension, as well as the optimal incubation time to assess the impact of bacteria on the change of gymnemagenin content in plant samples.

In preliminary studies, different portions of *sample preparation* (section 2.2.1) from sample G5/0 (containing 1.71% (w/w) of gymnemagenin, see Table 2) were incubated with either 20 mL, 50 mL, 70 mL, or 100 mL of *bacteria suspensions* from each strain of bacteria for 10 days. After incubation, the gymnemagenin content was determined by the HPLC method. Experimental results (Fig. 3, A) showed that the gymnemagenin content after incubation increased when the volume of bacterial suspension increased from 20 mL to 70 mL, but comparing gymnemagenin contents after incubation with 4 bacterial strains (*B. subtilis*, *B. bifidum*, *L. casei*, and *L. rhamnosus*) with 70 mL and with 100 mL of bacterial suspension, the difference was not statistically significant (P = 0.30 with paired t-test). From these results, it was clear that after 10 days of incubation, 70 mL of *bacteria suspension* was sufficient to transform all types of gymnemic acids available in the *sample preparation* into gymnemagenin, and when higher volumes of bacteria suspension were used, the surplus of bacteria produced no further increase of gymnemagenin because gymnemic acids in the sample had completely been hydrolyzed to form gymnemagenin. So in further experimental works, the ratio of 70 mL of *bacteria suspension* for one portion of *sample preparation* was consistently used.

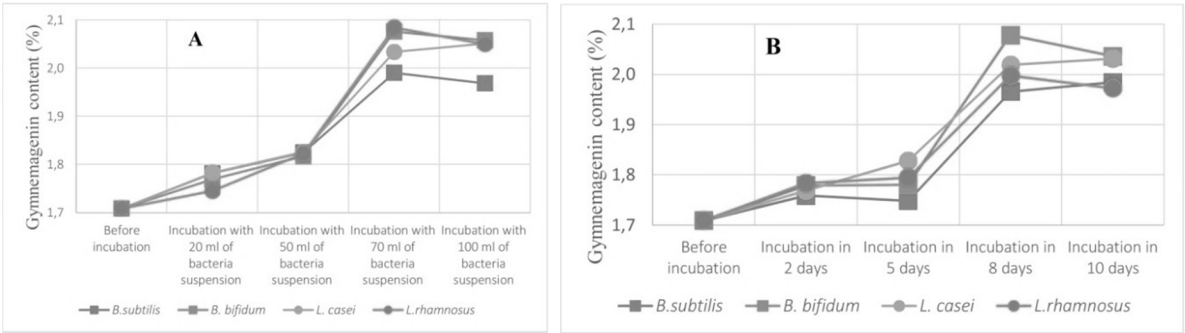

Fig. 3. Preliminary results on bacteria suspension volume (A) and incubation time (B).

In terms of incubation time, different portions of *sample preparation* were incubated with 70 mL *bacteria suspension* of each bacteria strain for 2 days, 5 days, 8 days, and 10 days.

Experimental results (Fig. 3, B) showed that the content of gymnemagenin increased when incubation time prolonged from 2 days to 8 days, but the gymnemagenin contents in sample obtained after incubation with the 4 strain of bacteria in 8 days and in 10 days did not differ significantly one from another (P = 0.58 with paired t-test), meaning no significant increase in gymnemagenin content was gained when incubation time went longer than 8 days. It meant that after 8 days, all the content of gymnemic acid in the sample preparation had been hydrolyzed to form gymnemagenin, and incubation times longer than 8 days were not necessary. So, the incubation time was fixed at 8 days for all further experimental studies.

These results also pointed out that with *a bacterial suspension* initially containing 10^8 CFU/ml, the content of gymnemagenin in samples began to increase fast only after 5 days of incubation. This observation was similar for all four bacterial strains, so the biotransformation rate to form gymnemagenin did not depend on the nature of the bacterial strain, but rather depended on the initial amount of bacteria in suspension and the growth rate of bacteria as a function of incubation time. However, it is necessary to carry out further study with different initial amounts of bacteria to find out more clearly the relation between the initial number of bacteria and the biotransformation rate in order to optimize the gain of gymnemagenin content and to reduce incubation time.

In this study, based on the results of preliminary studies, the incubation time was fixed at 8 days, and the volume of *bacteria suspension* was selected at 70 mL per *sample preparation* for further studies.

3.3. Evaluation the impact of bacteria on gymnemagenin content

After 8 days of incubation, gymnemagenin content of all 4 samples (both *G.sylvestre* ones and *G. latifolium* ones) was increased with all 4 strains of bacteria (Table 2 and Fig. 4). However, the mean value of gymnemagenin content calculated on the 4 tested samples after incubation with *B. subtilis* did not increase significantly (P = 0.07 with paired t-test). Therefore, within the scope of results obtained in this study, only *B.bifidum*, *L.casei*, and *L.rhamnosus* helped increase the gymnemagenin content in the tested samples significantly after incubation.

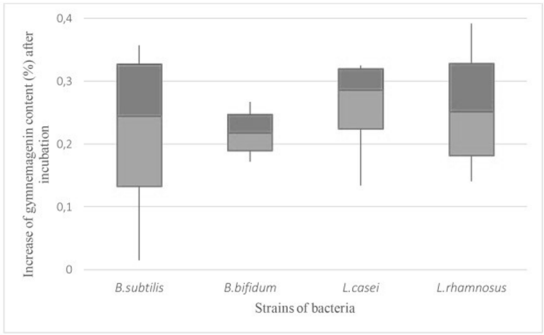

Fig. 4. Increase of gymnemagenin content (%, w/w) in samples after incubation

Table 2. Content of gymnemagenin in samples before and after incubation

Sample	***Gymnemagenin content (%, w/w)***				
	Before incubation	***After incubation***			
		B. subtilis	***B. bifidum***	***L. casei***	***L. rhamnosus***
G5/2/2/2a	2.11	2.28	2.28	2.36	2.30
G5/0	1.71	2.07	1.98	2.03	2.02
G4/11	1.81	1.82	2.00	1.94	1.95
G4/5	1.50	1.82	1.74	1.83	1.89
Mean values (%)	1.78	2.00	2.00	2.04	2.04
Statistical comparison of mean values of the content of gymnemagenin by paired t-test					
Before and after incubation with different strains of bacteria		P = 0.07	**P = 0.00**	**P = 0.01**	**P = 0.02**
Strain		***B. subtilis***	***B. bifidum***	***L. casei***	***L. rhamnosus***
After incubation with different strains of bacteria	***B. subtilis***		P = 0.96	P = 0.32	P = 0.33
	B. bifidum			P = 0.34	P = 0.41
	L. casei				P = 0.97
	L. rhamnosus				

Among these three strains, no significant strain-to-strain difference was observed in their capacity to increase the content of gymnemagenin in the tested *Gymnema* R.Br's samples (paired t-test yielded P values higher than 0.05 for all strain-to-strain comparisons, as presented in Table 2).

From these results, it is conclusive that *B. bifidum*, *L. casei*, and *L. rhamnosus* were capable of significantly increasing the content of gymnemagenin in *G. sylvestre* and *G. latifolium*

samples after 8 days of incubation at 35°C ± 2°C, and their capacities were not significantly different from one another. In contrast, *B. subtilis*'s capacity to increase gymnemagenin content was not significant in the tested samples. Among the four strains of bacteria, all are capable of producing lactic acid, but only *B. subtilis* is capable of forming protective endospores, and this characteristic can be a factor rendering *B. subtilis* less efficient in increasing the content of gymnemagenin than the other three strains, which are not capable of forming protective endospores and thus are not hindered by this transformation.

Experimental results also showed no significant difference in the capacity of *B. bifidum*, *L. casei*, *L. rhamnosus,* and *B. subtilis* to increase the content of gymnemagenin in identical incubation times. This might reflect the nonstrain-dependent nature of their capacity to increase gymnemagenin in contact with samples during the incubation period. However, a study on a larger scale involving more strains of bacteria and using more *Gymnema* R.Br samples would be necessary to arrive at more conclusive findings on the action mechanism of bacteria and more optimal conditions to employ bacteria for improving the gymnemagenin content in plant materials.

4. Conclusion

In this study, the capacity to increase the gymnemagenin content in *Gymnema spp.* R.Br samples of four strains of bacteria – *B. subtilis*, *B. bifidum*, *L. casei*, and *L. rhamnosus* – was investigated. When bacteria suspensions initially containing 10^8 CFU/mL were used, and the amount of tested samples was fixed at 0.75 g, the most suitable ratio for incubation was 70 mL of bacteria suspension for one portion of sample preparation, and the most suitable incubation time was 8 days.

Among the four bacterial strains, *B. bifidum*, *L. casei*, and *L. rhamnosus* were proven to be capable of increasing significantly the gymnemagenin content in samples of *G. sylvestre* and *G. latifolium* after 8 days of incubation at 35°C ± 2°C.

The relation between the incubation time and the increase of gymnemagenin content in the sample was found to depend on the initial amount of bacteria rather than on the nature of the bacterial strains. Experimental results also revealed no significant strain-to-strain difference in the capacity to increase gymnemagenin content in plant materials.

From the findings of this study, in order to further optimize the incubation efficiency and to increase the gymnemagenin content after fermentation, it is necessary to carry out further studies on the relationship between the initial amount of bacteria and the biotransformation rate to produce gymnemagenin during the fermentation process.

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