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(Original Article)

Molecular Characterization and Genetic Improvement of Some *Bifidobacterium* Isolates for Yogurt Manufacture use

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Abstract

Fermented dairy such as yogurt, have gained popularity due to their potential health benefits. These products contain probiotic bacteria, including *Bifidobacterium* species, which are known for their positive effects on gut health. Overall, this study aimed to isolate and molecularly characterize different isolates of probiotic bacteria and to enhance their fermentation time by mutagenesis. The local market and Faculty of Agriculture farm in Assiut University were used to collect different samples such as fermented dairy products, milk, and soybeans. These various sources were used to isolate different bacterial isolates.

Thirty-three different bacterial isolates were collected and identified biochemically, morphologically, and molecularly using PCR specific primers. Morphological, biochemical, and molecular tests showed that 13 isolates were identified as *Bifidobacteria* and further species identification were also done. Milk fermentation time was also tested for all the isolates. The fermentation time measurement showed that isolate no.11 had the lowest curd time (13 to 14 h). After mutagenesis, the mutant obtained from isolate No.11 recorded enhanced fermentation time of only 4 h.

Keywords: *Bifidobacterium sp., Fermentation time, Mutation, Probiotics.*

Introduction

Probiotic is a Greek word; “pro” means “in favor” and “biotic” means “life” (Mazhar *et al*., 2020). Probiotics are often referred to as “good bacteria” because they help maintain a healthy balance of microorganisms in the gut (Hill *et al*., 2014; Fuller *et al*., 1991). Nowadays, probiotic microorganisms have been added into food as dietary adjuncts for improving human health as well as prevention of many diseases (Moradi *et al.*, 2018). Probiotic bacteria play an important role in the protection of the organism against harmful microorganisms and strengthen the host’s immune system when administered in appropriate quantities *(*Pundir *et al*., 2013). There is plenty of evidence that some probiotics have many beneficial health effects, including suppressing harmful bacteria and metabolites in the intestine, and inhibiting intestinal disorders (Cain and Karpa, 2011). The use of

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probiotics and prebiotics in functional foods has gained popularity due to their potential to improve digestive health and overall well-being. Research has shown that these ingredients can help maintain a balanced gut microbiota, which is essential for proper digestion and nutrient absorption. Many probiotic strains have been added to various food products, such as yogurt, fermented food, and dietary supplements (Gomes and Malcata, 1999). These strains can include bacteria, such as Lactobacillus, Streptococcus and *Bifidobacterium* species, as well as certain yeasts which were found mostly in fermented dairy products (Hassanzadazar *et al*., 2012; Fijan, 2016). *Bifidobacterium* is one of the most used probiotic microorganisms in the food industry due to its health enhancing benefits (Singh *et al*., 2020). It was first isolated in 1899 from a healthy breast-feed infant by Tissier of the Pasteur institute in France (Tissier, 1899). *Bifidobacteria* are gram-positive, non-spore forming, non-gas producing, nonmotile, catalase-negative and anaerobic or microaerophilic bacteria. The most famous genera of *Bifidobacteria* used in probiotic foods *B. bifidum* , *B. longum* , *B. breve*, *B. angulatum* and *B. adolescentis* (Sgorbati *et al*., 1995).

When compared to other lactic acid bacteria utilized in fermented dairy products, *Bifidobacteria* development in milk is frequently restricted or delayed. This is because *Bifidobacteria* are more susceptible to environmental changes and have unique dietary needs. They need specific kinds of carbohydrates that are not easily found in milk, including oligosaccharides. Furthermore, *Bifidobacteria* are more delicate to oxygen concentration and acidity. The study conducted by (Yonezawa *et al*., 2010); and (Odamaki *et al*., 2011) investigated the growth of *Bifidobacteria* in skim milk was enhanced when *Bifidobacteria* was co-cultivated with specific strains of *Lactococcus lactis*.

Molecular identification of *Bifidobacterial* isolates can be achieved using specific primers that target specific regions of the bacterial genome *(*Matsuki *et al*., 2003). Genetic improvement of some *Bifidobacteria* isolates using UV rays to cause mutations (Abdel Ghany Elrahmany *et al*., 2023). The aim of this study is to isolate *Bifidobacteria* from natural sources such as fermented dairy products and some raw milk such as goat milk, camel milk, human milk and some natural products such as bee honey and soy, then define these isolates by chemical and morphological methods.

Material and Methods

Sample collection for bacterial isolation

Several samples (Table 1) such as honey, curd milk, goat milk, pickled whey, breast milk, camel milk, yogurt, and soybeans were used to isolate several bacterial isolates.

The collected samples were suspended in 100 ml of distilled water, then several dilutions were made. 100 µl of each dilution was pipetted onto a petri dish containing MRS agar containing 0.25% L-cysteine and incubated at 37°C for 48 hours in an anaerobic chamber (Vasudevan *et al*., 2021). Different colonies were

picked up individually repeatedly sub- cultured for purification and maintained on sterile MRS slants, coded and then stored at 4 °C until needed.

Identification of bacteria by biochemical tests

Several biochemical tests were done as prescribed in the Bergey's Manual of Systematic Bacteriology (Scardovi, 1986) to identify the *Bifidobacteria* isolates. These tests include; Catalase test, bacterial movement, hydrogen sulfide production, and Gram stain tests. Anaerobic, Catalase-negative, nonmotile, non-gas producing, Gram- positive isolates were selected as possible *Bifidobacteria* candidates for further investigation.

Molecular identification of *Bifidobacteria* isolates by using specific primers

DNA isolation

Genomic DNA was extracted from different bacterial isolates as described by (Saghai-Maroof *et al*., 1984) with some modification. 1 ml of overnight liquid culture was placed in a 1.5 ml disposable centrifuge tube. The cells were collected through centrifugation at 10000 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 0.2 ml of phosphate buffer. 20 µl of lysozyme were added and incubated at 370 C for 60 minutes. 0.4 ml of CTAB extraction buffer was added followed by 40µl of ß-mercabtoethanol and gently mix. The tube was placed in 60oC water bath for 30 min. After cooling an equal volume of Chloroform: isoamyl (24:1 v/v) were added and mixed vigorously. This mixture was then centrifuged for 10 min at full speed, and the aqueous supernatant was transferred to a new tube. An equal volume of cold ethanol (100%) was added then cooled at -4ºC for 30 min, then centrifugated for 5 min at 1300 rpm to pellet DNA. Washing was done with ethanol 70% followed by centrifugation for 5 min. Finally, the pellets were kept for drying for 1hr at room temperature and then dissolved in 100 µl of H_2O.

PCR reactions preparation

Molecular identification for all the isolates was performed using species-specific primers. Four different specific primers (Table 1) for *B. bifidum*, *B. longum* and *B. breve* identification were used to identify the different isolates.

Table 1. Used species-specific primers for Bifidobacterium.

Use	Name of primers	Sequence 5' to 3'	Product size(bp)	Tm©	Reference
Bifidobacterium	g-bifid-F	CTCCTGGAAACGGGTGG	549-563	51	Matsuki *et al*.,2002
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA		51	
B. bifidum	BiBIF-1	CCACATGATCGCATGTGATTG	278	51	Matsuki *et al*., 1998
	BiBIF-2	CCGAAGGCTTGCTCCCAAA		52	
B. longum	BiLON-1	TTCCAGTTGATCGCATGGTC	831	51	Matsuki *et al*., 1999
	BiLON-2	GGGAAGCCGTATCTCTACGA		52	
B. breve	BiBRE-1	CCGGATGCTCCATCACAC	288	52	Matsuki *et al*., 1998
	BiBRE-2	ACAAAGTGCCTTGCTCCCT		50	

PCR reactions were conducted in a 20µL total volume, containing 10x PCR master mix (GeneDirex), 1 µL of each primer (100 ng/ µL) and 2 µL of DNA template, and 7 µL dH2O. The PCR program was as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, 5oC of annealing, 60 s extensions at 72°C, and a final extension for 5 min at 72°C.

Gel electrophoresis

PCR products were electrophoresed onto a submerged 1% agarose gel and the results were compared with a 100 bp ladder marker. The gel documentation system was used to visualize and photograph the gels.

Determination of yogurt fermentation time caused by different isolates

To determine the yogurt thickens time, fresh milk (with 3% fat and 8.5% SNF) obtained from the dairy laboratory at the faculty of Agriculture, Assiut University was sterilized at 121 °C for 10 min followed by immediate cooling at 37 °C. Then, 9 ml of this milk was inoculated with 1 ml appropriate dilution (ensuring equal proportions of bacteria in each isolate) of isolate overnight culture, followed by incubation at 37 C. Different samples were monitored frequently (from time 0 to 24 h) to score the duration used to convert milk to yogurt for each isolate.

Enhancing the fermentation time of a *Bifidobacteria* isolated by mutagenesis

The mutation procedure aimed to induce genetic changes in the bacteria to potentially enhance it fermentation time. To mutate the isolates, five serial dilutions of the isolate were made (no. 11), and 100 µL each dilution were poured on a petri dish containing MRS medium supplemented with 25% L-cyctein. The plates were kept drying at room temperature. Then the plates were exposed to UV rays for different durations (0.25, 0.5, 1, 2, 3, 4 and 5 min). Plates were covered and incubated overnight under anaerobic conditions to produce mutants. 25 different mutants were chosen randomly and screened for their fermentations time as described earlier.

Results and discussion

Bifidobacterium isolation

Several samples such as; honey, curd milk, goat milk, pickled whey, human breast milk, camel milk, yogurt, and soybeans were used to isolate several bacterial isolates. A total of 33 different bacterial isolates were collected from various sources as shown in Figure (1).

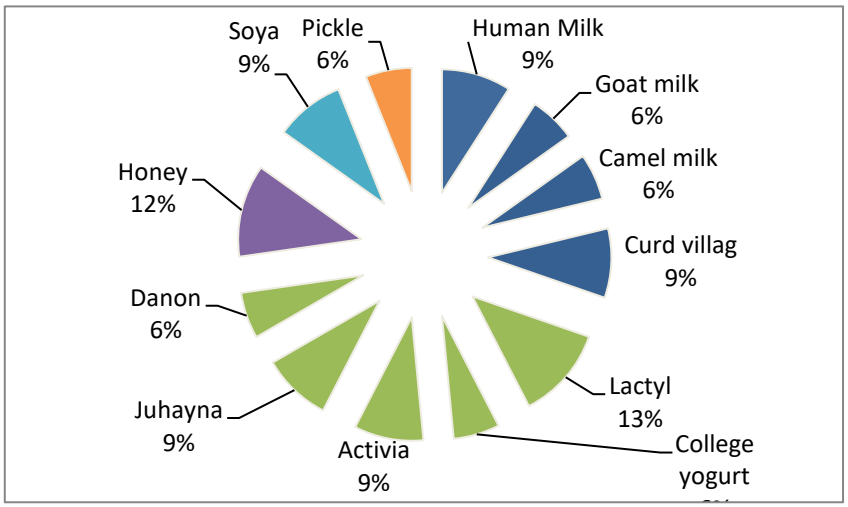

Figure 1. The percentage of the bacterial isolates from different sources.

The largest number of isolates was obtained from different types of yogurts, where the percentage reached about 43%, followed by different types of Milk, where the isolates percentage reached 30%. In third place comes honey, where the percentage of isolates reached 12%, then soybeans 9%, and finally pickles 6%.

Bacterial identification by biochemical test

The bacterial isolates were identified according to morphological and biochemical tests (Bergey's Manual of Determinative Bacteriology). Results (Table 2) showed that out of 33 bacterial isolates only 13 isolates could be identified as *Bifidobacteria*.

Table 2. Different sources of samples which were used for bacteria isolation.

Location	Samples	Total Number of isolates	*Bifidobacteria* isolates
Dairut city	Honey	4	1
	Curd milk	3	1
	Goat milk	2	2
	Pickled whey	2	2
	Human milk	3	1
Aswan	Camel milk	2	1
Assiut	Yogurt	15	3
Faculty of Agriculture farm	Soybeans	2	2
Total		33	13

Molecular identification of *Bifidobacterium* isolates by PCR

Molecular identification for *Bifidobacterium* isolates was performed using specific primers. Four different specific primers were used. The first primer (g-bifid) is a general primer for *Bifidobacterium* genus identification. All the 33 collected isolates were tested by this primer. The results in Figure (2) showed that only 13 isolates reacted with this primer and produced a band ranging in size between 549 bp and 563 bp. The *Bifidobacterium* identified isolates were isolates no. B1, B2, B3, B11, B12, B13, B14, B15, B21, B22, B23, B31, and B32. These isolates were the same isolates which were identified by the morphological and biochemical.

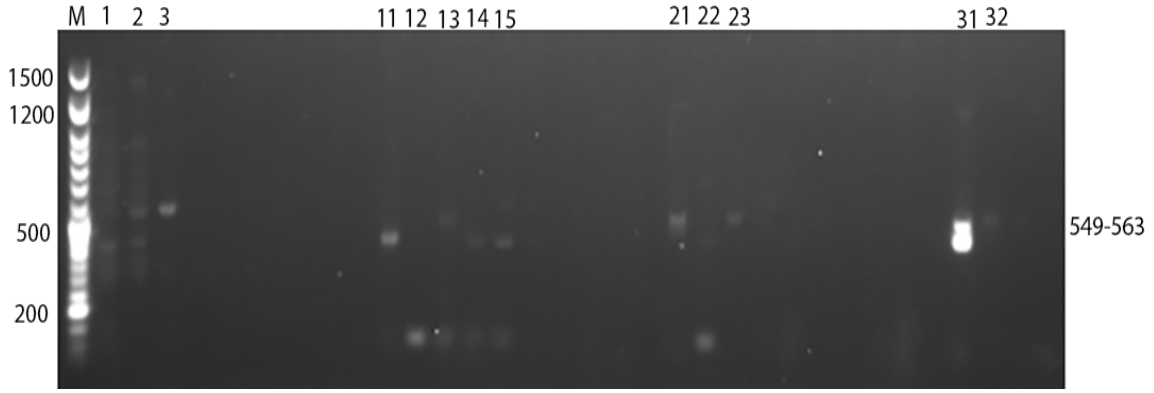

Figure 2. Agarose-gel electrophoresis of PCR products produced by the g-bifid primer for the identification Bifidobacterium isolates

With these findings we could confirm that these 13 isolates were *Bifidobacterium,* but the types of the species are still unidentified, so another 3 species-specific primers were used to identify the species.

For *B. bifidum* identification, a PCR reaction containing BiBIF primers, which produce a band with a size of 278 bp with *B. bifidum* isolates, was used to test only the 13 *Bifidobacterium* identified isolates. The results in Figure (3) showed that only isolates no. B11, B13, and B21 were able to amplify the expected band size, so these isolates were identified as *B. bifidum*.

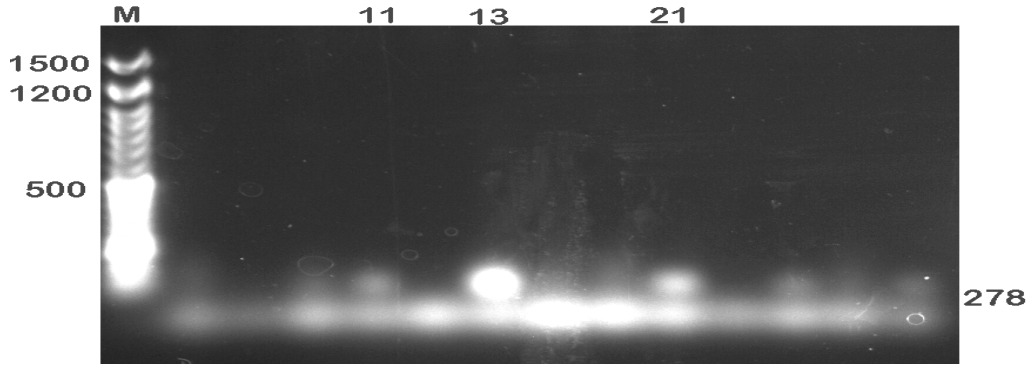

Figure 3: PCR products for the BiBIF primer used to define B. bifidum isolates

For *B. Longum* identification, a PCR reaction containing BiLON primers, which produce a band with a size of 831bp with *B. Longum* isolates, was used to test only the 13 *Bifidobacterium* identified isolates. The results in Figure (4) showed that only 2 isolates (no. B1, and B31) were able to amplify the expected band size, so these isolates were identified as *B. Longum*.

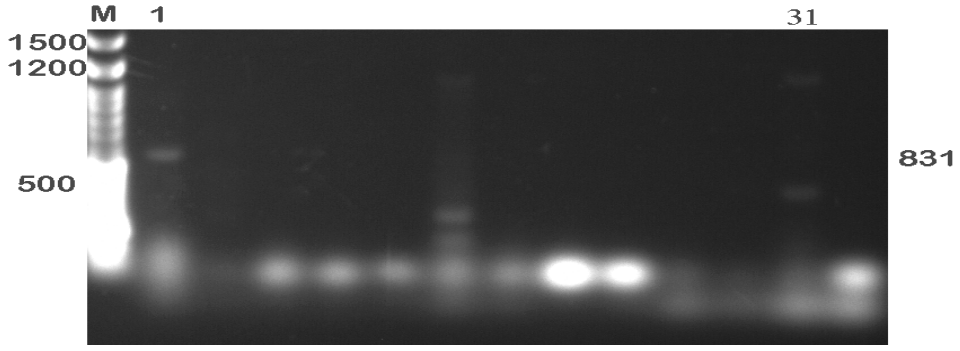

Figure 4: PCR products of the BiLON primer which were used to define B. longum isolates.

The BiBRE primer was used to identify the *B. breve* isolates in the 13 *Bifidobacterium* identified isolates. Surprisingly, none of the tested isolates produced the expected band (288 bp) which indicates that none of the isolates could be identified as *B. breve*.

Only five isolates were identified out of 13 isolates, and further research is required to identify other isolates that may be of the type *B. adolescentis*, *B. animalis*, *B. asteroides*, *B. dentium*, *B. ruminantium*, B. *merycicum*, or *B. tsurumiense.*

Determination of yogurt fermentation time caused by different isolates

The growth of *Bifidobacteria* in milk is slow or limited compared to other lactic acid bacteria used in fermented dairy. This is because *Bifidobacteria* have specific nutritional requirements and are more sensitive to environmental conditions. They require certain types of carbohydrates, such as oligosaccharides, which are not readily available in milk. Additionally, *Bifidobacteria* are more sensitive to acidity and oxygen levels, which can further limit their growth in milk. However, certain strategies can be employed to enhance the growth of *Bifidobacteria* in milk, such as prebiotic supplementation or optimizing fermentation condition (Champagne *et al*., 2005).

Figure (5) showed the results of the Fermentation time of the milk to which the isolated strains were added, the result showed that the 13 isolates that were positive for Gram stain and according to the biochemical definition are considered a *Bifidobacterium* strain, curing in a period ranging from 14 to 24 hours. The results, according to the fermentation test, showed that the best strain was No. 11 in terms of consistency.

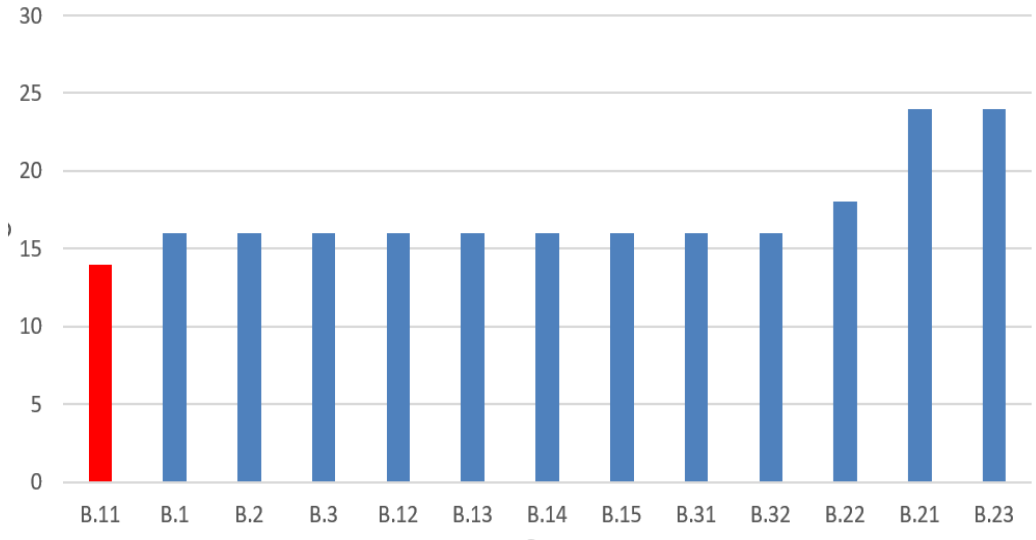

Figure 5: Fermentation time for each Bifidobacterium.

Determine the fermentation time for the milk to which the other isolates were added, as in Figure (6). The results showed that there were isolates that fermented in a record time of 10 h, but they were not identified molecularly.

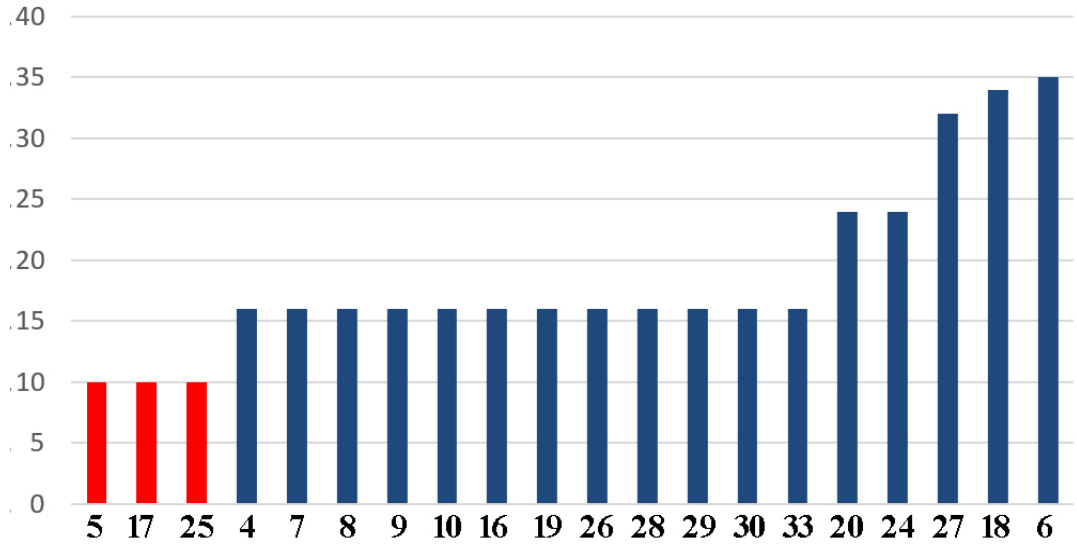

Figure 6. Fermentation time for other isolates

The mutation procedure

By treating the strains with ultraviolet radiation for different times, the best mutants strain in terms of curing speed were obtained in record time. Strain No.17 was cured after 4 hours, followed by strain No13 and 14, which were cured after 6 hours. The results of the experiment demonstrate the effectiveness of using ultraviolet radiation to induce mutations strains, leading to improved curing speed.

About 25 mutant strains (Figure 7), the best mutant strain was strain No.17, as it made the milk curd and, given the consistency of yogurt within a record time (4 hours) while creating the ideal desired consistency.

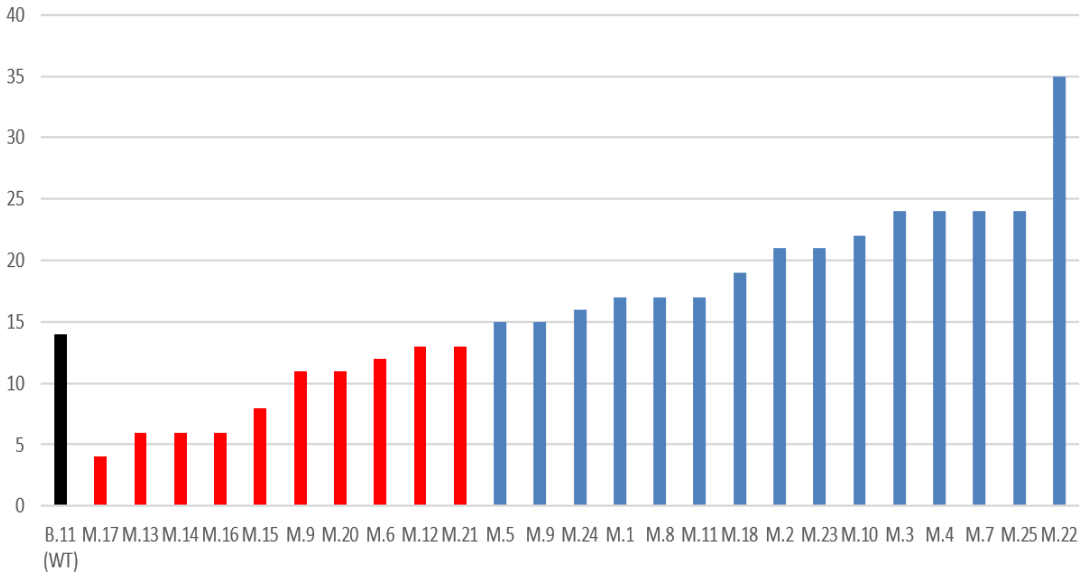

Figure 7. After manipulated by UV irradiation for different times

Conclusion

The purpose of this study is to collect isolates of *bifidobacterium* from different sources and then identify these strains biochemically as a preliminary definition. First, the bacterial strain was isolated from various sources. These strains were then subjected to biochemical tests to identify their characteristics and determine Their species. The results obtained can contribute to our understanding of bacterial diversity and evolution. The molecular identification of isolates using specialized primers was extremely important, in addition to the genetic improvement of some isolate by using UV at different times in order to improve the curing duration without the need for regular yogurt primers.

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التوصـيف الجزيئي والتحسـين الوراثي لبعض عزلات البيفيدوبكتيريا المسـتخدمة في صـناعة الزبادي

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الملخص

اكتسـبت منتجات الألبان المتخمرة مثل الزبادي شـعبية كبيرة بسـبب فوائدها الصـحية لما تحتويـه هذه المنتجـات على بكتيريـا بروبيوتيك، بمـا في ذلك أنواع البيفيدوبـاكتيريوم، المعروفـة بتأثيراتها الإيجابية على الصـحة. هدفت هذه الدراسـة إلى عزل والتوصـيف الجزيئي لعزلات مختلفة من بكتيريا البروبيوتيك وتحسين قدرتها على احداث التخمرات عن طريق استحداث بعض الطفرات. تم تجميع عينات مختلفة مثل منتجات الألبان المتخمرة والحليب وفول الصـويا من أماكن مختلفة مثل مزرعة كلية الزراعة بجامعة أسيوط وتم استخدام هذه المصادر لعزل سلالات بكتيرية مختلفة. تم تجميع ثلاثة وثلاثين عزلة بكتيرية مختلفة وتم تعريفها جزيئيا باسـتخدام البادئات متخصصة. أظهرت الاختبارات المورفولوجية والكيميائية الحيوية والجزيئية أنه من ضمن الـ 33 عزلة أمكن الحصول على 13 عزلة مختلفة من بكتيريا البيفيدوباكتيريا. أظهرت نتائج اختبار قدره العزلات على احداث تخثر أن العزلة رقم 11 كانت اكفاء العزلات في احداث التخثر (13 إلى 14 سـاعة). كما أظهرت نتائج الاسـتحداث بالطفرات حدوث تحسـن وتقليل مده التخثر في بعض الطافرات حيث وصل زمن التخثر الي 4 ساعات فقط.