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The Effect Of Celiac Disease On The Numbers And Types Of Bifidobacterium Spp. In The Patients Of AL, Muthanna Province-Iraq.

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Abstract

Objective: To identify number and types of Bifidobacterium spp. of the celiac disease patients.

Patients and methods: A prospective case series study was conducted at private clinics in Al-Muthanna city during the period from July 2020 to March 2021. A total of 75 patients (35 males, 40 females) and total of 25 healthy individuals (13 males, 12 females). The identification of Bifidobacterium sp. was carried out by morphological standard of colonies, microscopic test, biochemical examinationand PCR technique using genus specific primer.One hundred samples (children , adolescents, adults and elderly) from three to fifty five years were enrolled in this study, including seventy five celiac patients and twenty five healthy individuals as control group, during the period from July 2020 to March 2021.

Results: The isolates showed gram positive reaction, they appear under the microscope as short rods, some curved, others Y-shaped, it may be single or dual. Some biochemical tests was done for diagnosisBifidobacterium in pure isolates, all of them were negative for (Catalase, Oxodiase, Gelatinase, Indole production, Voges-Proskauer, and Production of ammonia from arginine). It is found that there is a significant decrease ($P < 0.05$) in Bifidobacterium counts in the celiac patients gut (1.1×10^5 cfu/gm) comparing with the Bifidobacterium counts in the control group (2.1×10^9 cfu/gm).Uniplex-PCR products ofBifidobacterium species using 16SRNA-specific primer. Showed the following species:Bifidobacteriumadolescentis (4), B. angulatum (7) The distribution of bifidobacterial species showed that theB. angulatum(22.7%), and B. adolesceatis(5.3%) in patients group.

Keywords : Celiac disese, Bifidobacterium,Uniplex**,** angulatum**,** adolesceatis

Introduction

Celiac disease is a genetically predisposed disorder characterized by villous atrophy, crypt hyperplasia, and lymphocyte infiltration in the small intestine in response to gluten-containing foods such as wheat, rye, oats, and barley (1).Celiac disease, on the other hand, is a systemic ailment that can affect organs other than the small intestine, including the colon, thyroid, skin, pancreas, and liver (2).Gluten's immune reactivity progresses over time, resulting in pathological alterations (3). The global prevalence of celiac disease in the general population is estimated to be between 1% to 2% (4). Celiac disease develops as a result of damage to the mucosa of the upper small intestine caused

by gluten (5). The word "normal microbial flora" denotes the population of microorganisms that reside in normal healthy individuals' skin and mucous membranes. Human beings are known to have approximately 10^{13} cells in their bodies and about 10^{14} bacteria are associated with them, most bacteria are found in the large intestine which is the normal flora (6). The bacterial distribution varies greatly at differentlevels of the gastrointestinal tract (GIT)ranging from $<10^{3}$colony-forming units/ml (CFU/ml) in the stomach to (10^{11}–10^{12} CFU/ml) within the colon, where anaerobes outnumber aerobes by a ratio of 1000:1(7).The study of this data has helped to uncover the dynamic relationship between the microbiota and the host. Literature has shown that changes in the proportion of these microorganisms can be related to pathologies that affect humans (8,9).The gut microbiota genome maintains normal physiological and metabolic functions in the human body, aiding food digestion through the metabolization of carbohydrates, vitamins, short-chain fatty acids and amino acids by significantly enriching genes (10).The genus Bifidobacterium, aindividual ofthe Bifidobacteriaceae family, belongs to the Actinobacteria phylum (11).To date, species included in the genusBifidobacterium are 29: Bifidobacterium adolescentis,B. angulatum, B. animalis(with the two subspecies B. animalis subsp. animalis andB. animalis subsp. lactis), B. asteroides,B. bifidum (type species), B.boum, B. breve, B.catenulatum, B. choerinum, B. coryneforme, B. cuniculi, B.dentium, B. gallicum, B.gallinarum, B. indicum, B. longum, B. magnum, B. merycicum, B. minimum, B. pseudocatenulatum, B. pseudolongum(with the two subspecies B. pseudolongum subsp.pseudolongum and B. pseudolongum subsp. globosum),B. psychraerophilum, B. pullorum, B. ruminantium, B. saeculare, B. scardovii, B. subtile, B. thermacidophilum (withthe two subspecies B.thermacidophilum subsp.thermacidophilum and B. thermacidophilum subsp. porcinum) and B. thermophilum (12).

Material and methods

Subjects

One hundred children , adolescents, adults and elderly from three year to fifty five years were enrolled in this study, including seventy five celiac patients and twenty five healthy individuals as control group.All patients in the study were referred and diagnosed by serological marker in the celiac in Al-Hussein teaching hospital in Al-Muthanna and in Al-Muthanna children's Hospital and some specialized medical clinics in Al-Muthanna.

stool sample collection

Stool samples were collected in clean and sterile contain thioglycolate brothfrom the celiac patients and healthy individuals at time of study , all Samples were transported to the laboratory and examined for the Bifidobacterium.

Classification Criteria:

The seventy five celiac patients were classified into two groups according to the gender as follows:

1. Group female (F): Forty patients.
2. Group male (M): Thirty five patients.

The twenty five healthy individuals were classified into two groups according to the gender as follows:

1. Group female (F): Twelve healthy individuals.
2. Group male (M): Thirteen healthy individuals.

list of culture media:

NO.	Culture Media	Company	origin
1	Bifidobacterium Agar, Modified	HIMEDIA	UAS
2	Fluid Thioglycollate Medium	LIOFILCHEM	UK
3	MR-VP medium	Oxiod	England

Methods:

Culturing of stool samples :

Under anaerobic condition using a anaerobic jar system,stool samples were cultured onBifidobacteriumagar by streaking to gate pure colonies of Bifidobacterium spp. .The isolates was subjected to some morphological and biochemical test for primary identification, than was sub cultured on slant for maintenance.

Enumeration of Bifidobacteriumin stool:

The Bifidobacterium were enumerated using the pour plate technique. Samples were used directly and also diluted to 10^{-1}, 10^{-2} to10^{-6}using normalsaline (13).Serial dilution is the process of diluting a sample with a sterile diluent, in a series of standard quantities. Then, using a small measured volume of each dilution, a series of pour or spread plates are created, it is possible to generate an incubated plate with an easily countable number of colonies (30–300) and compute the number of microorganisms present in the sample (13). Calculation the number of Bifidobacterium in the sample according to (14).

Total number = average of microbes in the selected dil. ×reciprocal of selected dilution

Morphological Characterization :

The isolates were observed with microscope for Gram staining. Gram staining was used to establish the morphological features of various isolates.Strains were observed with a microscop (100 x) after coloration with methylene blue, it was diagnosed according to the approved diagnostic sheet. Colony characterization was performed, such as shape, size, surface, elevation and edge formation of the colony (15).

Biochemical tests :

Final identification is done through using biochemical tests which include Catalase test, Oxidase test, Gelatinase test, Indole production test, Methyl red test, Voges-Proskauer test, and Production of ammonia from arginine, all this assay was carried out according to the (16, 17,18, 19, 20, 21) respectively.

Molecular tests:

Primer Design : primer of the Bifidobacteriumspecies :

The oligonucleotide primers for all genes used in this study were obtained from previous studies (22).Oligonucleotide primers were synthesized by Macrogen company (Korea). All genes used in the present study were summarized in table (1-1).

Table (1-1) Bifidobacteriumspecies- and group-specifc primers based on 16S rRNA sequences:

Name of primers	Sequence[a]	Length	Target site[b]	Product size	Aimed human intestinal bifdobacteria
BiADO-1	**CTCCAGTTGGATGCATGTC**	**19**	**182-200**	**279**	**B. adolescentis**
BiADO-2	**CGAAGGCTTGCTCCCAGT**	**18**	**476-442**		
BiANG-1	**CAGTCCATCGCATGGTGGT**	**19**	**185-203**	**275**	**B. angulatum**
BiANG-2	**GAAGGCTTGCTCCCCAAC**	**18**	**476-441**		

DNA Extraction:

According to the instruction of manufacturer company(Geneaid).

PCR Assays for Identification of Bifidobacterium species :

In this study, PCR assays (Uniplex) were used to confirm identification of Bifidobacterium by using specific genes.This experiment was carried out with modest changes to the PCR mixes and conditions. After PCR amplification of DNA in a final reaction mixture volume of (25 µl) for 30 cycles, the results of PCR amplification were electrophoresed on a (1.5%)agarose gel and observed under UV-transilluminator. PCR mixtures and PCR conditions of this assay was summarized in Table (1-2).

Table (1-2) Uniplex PCR mixtures and conditions for identification of (BiBIF, BiCATg, BiADO, BiANG, BiBRE and BiLONg) gene.

PCR mixtures		**PCR conditions**		
PCR Master	**Volume**	**Steps**	**Condition**	**No. of cycles**
Master Mix	12.5µl	Initialization	95 °C for 5 min	1
Forward Primer	1.3 µl	Denaturation	94 °C for 1 min	30
Reverse Primer	1.3 µl	Annealing	**55**-57.8 °C for 1 min	
DNA template	3.0 µl	Extension	72 °C for 1 min	
Nuclease-Free Water	6.9 µl	Final Extension	72 °C for 10min	1

Statistical analysis :

Data were processed by using statistical program social science (SPSS 22).T test was used to find the P value of antiradar markers. The level of significance was 0.05 (or less) in all statistical testing, (p value less than 0.05).Significance of differences were evaluated by Fisher's exact test and chi square test. A P value of ≤0.05 was considered statistically significant (23).

Results:

Microbiology tests :

A total of 100 Bifidobacterium isolates were isolated and identified from feces, of75 Celiac patients and 25 healthy individuals.The species identified was (B. adolescentis, and B. angulatum,). The diagnosis based on morphologicaland biochemical tests, as well as using confirmated by Polymerase chain reaction (PCR).

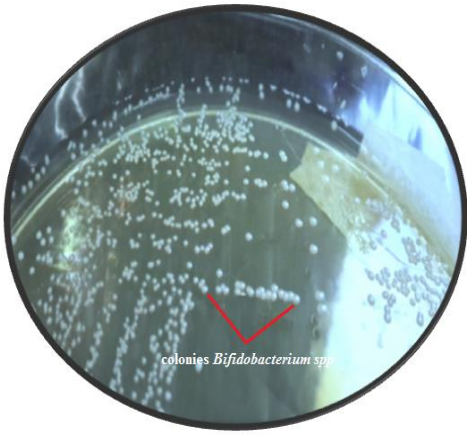

Fig (1-1) : Colony*Bifidobacterium spp*. on the (*Bifidobacterium* Agar, Modified)

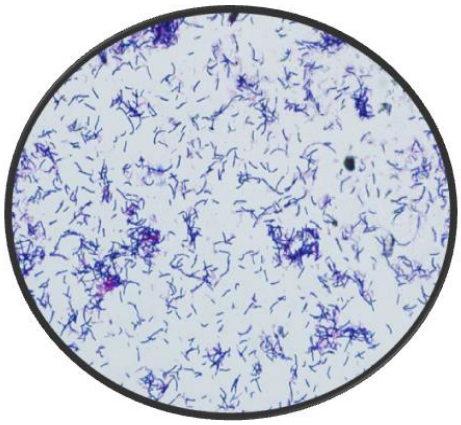

Fig (1-2) : *Bifidobacterium spp* cells under a light microscope (100X), and using Gram stain

Table (1-3) shows some biochemical tests for diagnosing Bifidobacterium in pure isolates, all of them were negative for tests (Catalase, Oxodiase, Gelatinase, Indole production, Voges-Proskauer, and Production of ammonia from arginine).

Table (1-3) Biochemical characteristics of the isolated species from Bifidobacterium

SpeciesBiochemicaltest	B. adolescentis	B. angulatum
Gram stain	+	+
Growth Anerobic	+	+
Catalase test	-	-
Gelatinase test	-	-
Indole production test	-	-
Oxodiase test	-	-
Production of ammonia from arginine	-	-
Voges-Proskauer test	-	-
Methyl red test	-	-

Comparison of Bifidobacterium counts between celiac patients and control group :

In table (1-4) it is found that there is a significant decrease (P < 0.05)in Bifidobacterium counts in the celiac patients gut (1.1×10^5 cfu/gm) coloniescomparing with theBifidobacterium counts in the control group (2.1×10^9 cfu/gm) colonies.

Table (1-4):A comparison between the count of Bifidobacterium in the celiac patientsand control group:

Count Bifidobacterium cfu/gm	Patients n = 75	Control n = 25
Mean	1.1×10^5 a	2.1×10^9 b
± SE	1.2×10^3	4.5×10^7
p - value	<0.0001	

The same letters indicate non-significant difference between groups but different letters indicate significant between groups, based on t-test.

Molecular identification:

Uniplex PCR Identification of Bifidobacteriumspecies strains:

To differentiate among Bifidobacteriumspecies, Uniplex-PCR products obtained with Bifidobacterium speciesstrains using 16S rRNA-specific primer.

Where 279bp product represents a B. adolescentis. Lanes (1, 4, 5, 6) represent the identified B. adolescentis; other lanes are negative. Lane M represent 100bp DNA ladder. as shown in figure (1-3).

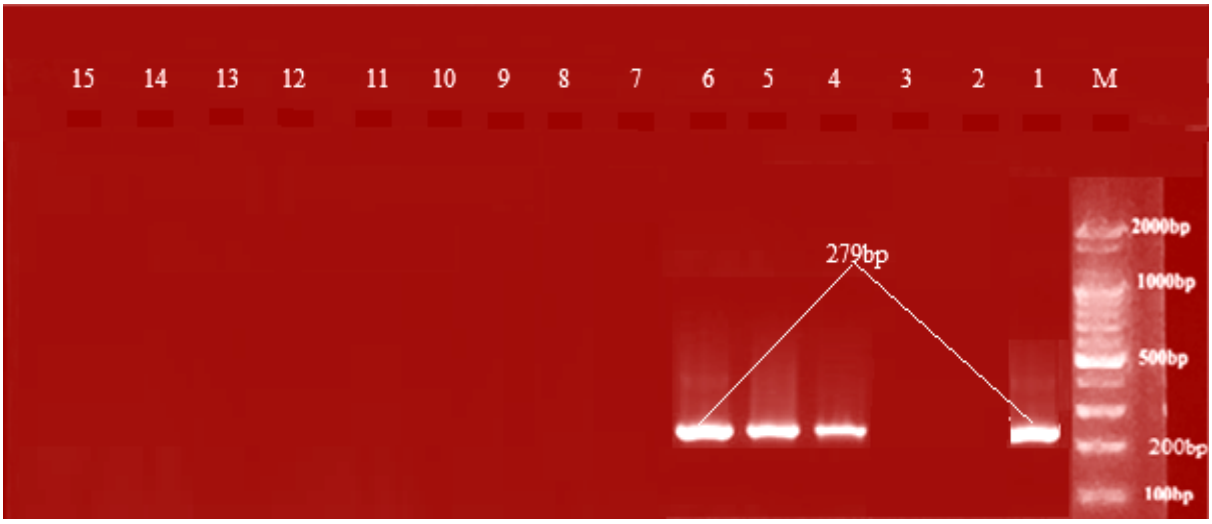

Fig. (1-3): Agarose gel electrophoresis of Uniplex-PCR products obtained with Bifidobacterium species strains using 16S rRNA-specific primer, where 279bp product represents a B. adolescentis. Lanes (1, 4, 5, 6) represent the identified B. adolescentis, other lanes are negative. Lane M represent 100bp DNA ladder. Note: (this is the first run of 15 samples only)

The B. adolescentis was lower in the isolated percentage of the patients with Celiac disease comparison the isolated percentage of the healthy, and significant difference at (P<0.05). The isolated of the positive are values were lower than the isolated of the negative values with a

significant difference at (P<0.05), where molecular methods confirmed the results that described in Table (1-5).

Table (1-5): Distribution of B. adolescentis among isolationof healthy and patients with Celiac disease.

Results	Positive	Negative	P value[a]
	No. (%)	No. (%)	
Healthy	13 (52.0%)	12 (48.0%)	<0.0001*
Patients	4 (5.3%)	71 (94.7%)	
P value[b]	0.029*	**<0.0001***	

* represent a significant difference at P<0.05.

When 275bp product is represents a B. angulatumlane (1,4,5,6,7,10,11,12,14,15) represents the identified B. angulatum; other lanes are negative. Lane M represent 100bp DNA ladder. as shown in figure (1-4).

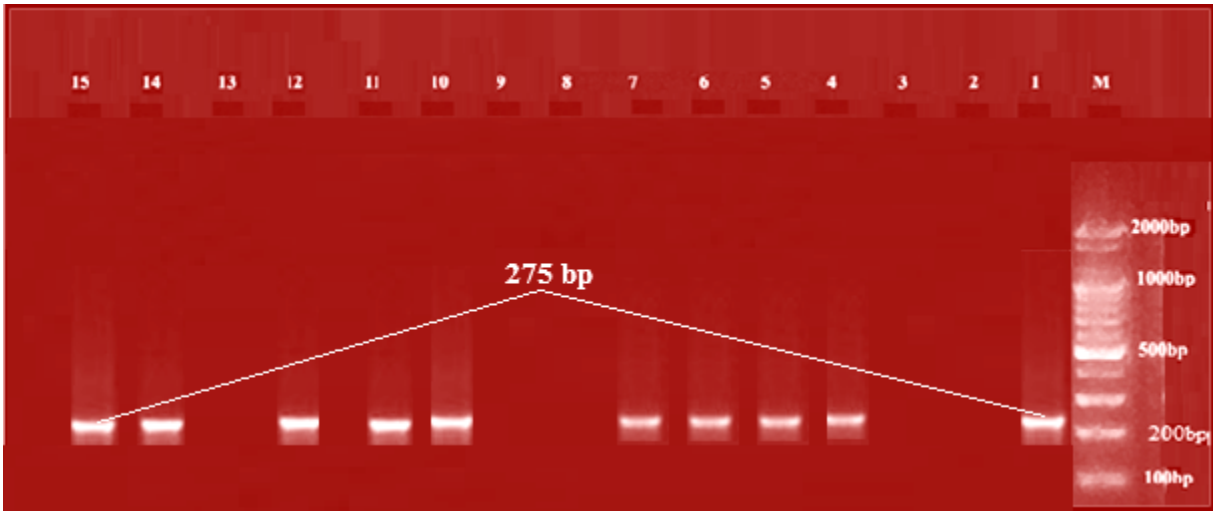

Fig. (1-4): Agarose gel electrophoresis of Uniplex-PCR products obtained with Bifidobacterium species strains using 16S rRNA-specific primer, where 275bp product is represents a B. angulatum. lane (1,4,5,6,7,10,11,12,14,15) represents the identified B. angulatum; other lanes are negative. Lane M represent 100bp DNA ladder. Note: (this is the first run of 15 samples only).

The isolated percentage of B. angulatum from patients were more than of the isolated percentage of healthy and significant difference at (P<0.05). The no-significant values were at (P>0.05) between the isolated positive are values and the isolated negative values . Table (1-6).

Table (1-6): Distribution of B. angulatumamong isolates of healthy and patients with Celiac disease.

Results	Positive	Negative	P value[a]

	No. (%)	No. (%)	
Healthy	2 (8%)	23 (92.0%)	0.087
Patients	17 (22.7%)	58 (77.3%)	
P value[b]	0.001*	**<0.0001***	

* represent a significant difference at P<0.05.

Distribution of Bifidobacterium species among healthy and patients with Celiac disease.

The results of an examination of bifidobacterium species distribution showed that theB. angulatum (22.7%), and B. adolesceatis (5.3%)in patients group, as shown in (fig. 4-9).

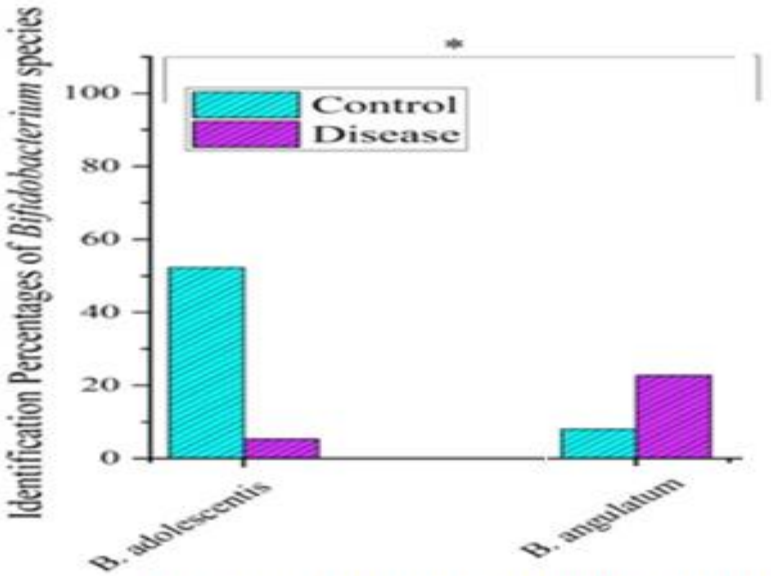

Fig. (4-9): Distribution of *Bifidobacterium* species among healthy and patients with Celiac disease.

Discussion:

Comparison of Bifidobacterium counts between celiac patients and control group :

Show bacterial cultures that the fecal Bifidobacterium count in the patients group lower compared to the facial Bifidobacterium in the control group. These results were in agreement with many researches including (24), who have stated there is decreased count of Bifidobacteria were reported in CD as compared to healthy personnel, which have effects exerted on gliadin caused by inflammation.

According to (25), proportions of Bifidobacteriumspp., Bifidobacteriumlongum, are lower in untreated CD patients than in healthy controls, our results match them.The results of this study matched what he pointed out (26) who stated that patients with celiac disease show a decrease in species Bifidobacterium and Lactobacillus and an increase in those potentially pathogenic (E. coli and Bacteroides) as compared to group healthy individuals.Our study also matched (27) views who have stated the celiac patients has higher damagely bacteria as Gram-negative bacteria: (Bacteroides, Prevotella, Escherichia coli) and fewer helpful bacteria as Gram-positive bacteria

including the protective Lactobacilli and Bifidobacterium.In addition, our study is consensus with (28) they found in their research that Firmicutes spp., Bifidobacterium, the anti-inflammatory bacteria, are decreasing in abundance, while Proteobacteria spp., which change metabolic function, are increasing.

Our study with matched the views of (29) and (30) who have stated there is, dysbiosis (reduction in microbial diversity) in CD is characterized by a decrease in Bifidobacteria numbers, according to multiple prior investigations.We believe the reason for the decline in Bifidobacterium numbers may be in the celiac patients' mucosal layer fails to stabilize the gut microbiota and protect the host from damaging antigens and infections, this conclusion corresponds to what (31) pointed out in their study.

Molecular identification:

Distribution of Bifidobacterium species among healthy and patients with Celiac disease.

Bifidobacterium species are thought to be important biological indicators of intestinal health. The human gut microbiota is guaranteed by common Bifidobacterium species. B. adolescentis, B. bifidum, B. breve, B. dentium, B. longum, B. angulatum, B. catenulatum, and B. pseudocatenulatum (32). catenulatum was higher in controls than in active and non-active CD patients.Also these findings are consistent with (35) who have stated the most predominant bifidobacterial groups detected in faeces, were B. catenulatum followed by B. breve.These findings are also in line with (22), who reported that a test of bifidobacterial species distribution revealed that the B. catenulatum group with healthy people accounted for 92 percent of the total.It's most likely the reason for the rise in predominance of the B. catenulatum group in the most regularly utilized specimens in the human adult study, this was also confirmed by (32) in their study who have stated the showed that B. catenulatum and B. pseudocatenulatum are members of the human adult intestinal microflora.

The results of the current study showed that there is significant decrease of the percentages of Bifidobacteriumadolescentisin the CD patients, comparison with percentages of Bifidobacterium species in the control, these results bring into line with other studies such as (33) who reported that the B. adolescentisprevalence was higher of controls than in those of active and non-active CD patients .

These findings differ from those of (34), who found B. adolescentis in slightly larger levels in stool samples of CD patients than in controls. This is most likely due to me. It's possible that the disparity is related to the employment of different identifying methods.

In comparison to percentages of Bifidobacterium species in the control group, the current study found a significant increase in the percentages of Bifidobacterium angulatum in CD patients. These findings are consistent with other studies (36) who found the Differences in faecal bacteria populations and faecal bacteria metabolism in healthy adults and celiac disease patients, increase percentages Bifidobacterium angulatumin the untreated vs control.

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