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Isolation bacteria from soaked chickpea using for bakery fermentation

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Abstract: *The aim of the present study was to isolate and determine the dominant and effective bacterial species present in chickpea soak which is used in Syria and Middle East for fermentation during the preparation of the popular Kaak pastry as well as other baked products.Thirty isolates were isolated from three samples of soaked chickpea collected from a local bakery store in Aleppo. Of these, 19 isolates were aerobic and 11 isolates were anaerobic. All isolates were tested for biochemical tests (sugar fermentation, catalase, indol, nitrate, VP, starch hydrolysis and growth at 6.5 % NaCl). Isolates were then tested by P1/P2 primers and results showed a single band at about 1100 bp. The GenBank database was then used to compare 16S rDNA sequences and there were three bacterial species found: Clostridium (*Clostridioides *Prazmowski 1880) sartagoforme, Bacillus thuringiensis (Berliner 1915) and Enterococcus faecium ([Orla-Jensen 1919] Schleifer & Kilpper Bälz 1984). Each isolated species was then tested solely for dough fermentation and the results were compared with the control (fermented using soaked chickpea mixture). The results showed that Clostridium sartagoforme. isolate gave a very similar fermentation power as the control (chickpea soak), although incubated for a longer time. As the significance and impact of study, Clostridium sartagoforme. strains isolated from chickpea soak can be used for production of this famous traditional pastry in Syria and Middle East to avoid the problems of the very short shelf life of the soaked chickpea and instability of its properties according to the success of soaking process.*

Keyword: *Bacillus thuringiensis, Clostridium sartagoforme, dough, Enterococcus faecium, kaak starter*

Introduction

A traditional type of rusks is being made by using water from fermented chickpea seeds as a leavening agent [1]. This pastry is produced as follows: coarsely ground chickpea. Seeds are put in a glass pot and then a certain amount of boiled water is added. The soaking water is slightly salted with NaCl 0.5% (w/v) and added to sample at 10: 1 (v/w)[2]. The pot is then incubated at 40-42^{0}C for fermentation. After about 12-14 h, when the foam has been formed like long hose, the content of the pot is thoroughly mixed with bread flour and the formed dough is kept warm for leavening to reach about twice of its original volume. Subsequently, the dough is mixed with more flour, warm water, sugar, ghee and some natural flavors to produce Kaak which is a famous traditional pastry in Syria and Middle East. The shelf life of this product is about six month at room temperature. The main problem in manufacturing of this product is the very short shelf life of the soaked chickpea

water which is about two days at 4^0C, also the product does not have stable properties in terms of taste and texture according to the success of soaking process, and this means a big loose for the manufacturer. If we know about the bacteria or yeast giving Kaak its special properties like flavor and structure, it will be much easier to stimulate the growth of this specific species and inhibit others during the soaking process, to get a special starter for Kaak instead of traditional way avoiding the variability between batches.

Materials and Methods

2.1 Chickpea seeds

bought from Aleppo local stores, were coarsely grounded using a mortar and pestle and then soaked in boiled tap water with 0.5% (w/v) salt added (w/v: 2/10) in a glass vessels. The vessels were incubated at 42^0C for 14 h.

The method used to prepare the fermented chickpea extract was similar to the traditional process, in respect to the relative proportions of chickpea and water as well as the fermentation time and conditions. Five replicates of soaked chickpea were done. The pH was measured directly for soaked chickpea water during fermentation using pH-meter (consort, Belgium).

2.2 Bacteria isolation

SPS medium (Merck) was used for isolation of *Clostridium sp*. Petri dishes were incubated anaerobically in anaerobic jar (Merck) with anaerobic bags (Merck) at 40°C for 48 h. M17 medium (Himedia) was used for lactic acid bacteria using the same previous procedure[3] . Nutrient agar (NA) (Merck) plates were incubated aerobically at 37°C for 48 h for isolation of aerobic bacteria.

2.3 Rheological Properties of Bread Dough

Farinograph and Extensograph were used to determine different rheological properties of dough. These tests were performed in triplicates according to AACC methods [4]. The Farinograph tests included: water absorption (amount of water required for the dough to have consistency of 500 Brabender units line), arrival time (the time in minutes required for the curve to reach the 500 Brabender unit line after start mixing with water), mixing time (the time in minutes from the first addition of water to reach dough's maximum consistency), stability (the time in minutes elapsing when the top of the curve interacts first 500 Brabender units line leaves that line), softness of wheat flour dough and its blends with pomegranate peel. 300 grams of tested samples (14% moisture basis) were used.

The Extensograph test was carried out to measure the following properties: dough extensibility (E) (the total length of the base of the Extensogram measured in millimeters), dough resistance to extension (R) (the height of the Extensograph curve was measured in Brabender units after 5 minutes from the start), dough energy (represented by the area in cm^2 out lined the curve)

and the peak height (the maximum height of the Extensograph curve measured in Brabender units) [5]

2.4 DNA isolation and identification from bacteria

Genomic DNA was prepared by using the following procedure: 10 ml overnight cultures were prepared in MRS broths. Cells were harvested in a micro centrifuge for 5 min at 6000 rpm. The pellet was then suspended in 200 µl 1xTE buffer (pH 8) containing 25% sucrose and 30 mg/ml lysozyme. The cell suspension was then incubated for 1 h at 37ºC. After the incubation, 370 µl, 1x TE (pH 8) containing proteinase K (1mg/ml) and 30 µl, 10% SDS were added. The samples were then incubated for 1 h at 37°C. Cells were lysed by the addition of 100 µl 5M NaCl and 80 µl CTAB/NaCl solution (10% cetytrimethylammonium bromide and 0.7 M NaCl), respectively. Lysed samples were incubated for 10 min at 65°C.

Chloroform extraction was performed twice using chloroform (chloroform/isoamyl alcohol: 24/1). First, one equal volume of chloroform/isoamyl alcohol was added and the samples were centrifuged for 5 min at 6000 rpm. The aqueous phase was then transferred into a new Eppendorf tube and Chloroform extractions were performed twice. The aqueous phase was transferred into a clean Eppendorf tube and the genomic DNA was precipitated by the addition of isopropanol (one equal volume). After that, precipitated DNA was washed using 500 µl 70% ethanol. When DNA precipitate was not visible, isopropanol containing samples were centrifuged for 10 min at 6000 rpm to pellet genomic DNA. After washing, DNA was pelleted by centrifugation for 10 min at 6000 rpm. Ethanol was removed and the pellets were air dried for 10 min at 37°C. Dried pellets were dissolved in 100 µl 1xTE containing 100 µg/ml RNase.

After incubation for 1h at 37°C, the sample volume was adjusted to 400 µl with 1xTE. DNA was dissolved by alternating cold-heat shock (80°C, 10 min to -20°C, 20 min as twice). DNA was further purified by phenol/chloroform extraction. One volume of phenol was added, and mixed well. After the centrifugation for 5 min at 6000 rpm, the aqueous phase was transferred into a fresh Eppendorf tube. Equal volume of chloroform/isoamyl alcohol was added and mixed well. Samples were centrifuged for 5 min at 6000 rpm. After that, DNA was precipitated by adding 1/10 sample volume of 5M NaCl and 2 volumes of 99% ethanol. Pellet was washed in 500 µl 70% ethanol by centrifugation for 5 min at 6000 rpm. After that, ethanol was removed and samples were dried for 10 min at 37°C. Finally, according to pellet size, it was dissolved in 50 µl, 100 µl or 150 µl 1xTE. Cold heat shock (for 20 min at 80°C and 20 min at -20°C) was performed in order to dissolve the samples. Dissolved genomic DNA samples were stored at -20^0C [6] DNA samples were tested for purity by spectroscopy the A260/A280 ratio and these samples were tested also by spectroscopy at A260 for the concentration of DNA. [7]

2.5 Amplification of 16S rDNA by PCR and sequence determination

Genomic DNA was prepared, as mentioned above, and 16S rDNA gene fragments were then amplified by PCR using the following universal primers: p1 (5'-

GCGGCGTGCCTAATACATGC-3') and p2 (5'-GGGTTGCGCTCGTTGCGGGA-3') which amplify the maximum number of nucleotides in 16S rDNA from a wide variety of bacterial taxa [8] [9]

The following program was used for amplification: 5min denaturation at 94^0C; 30 subsequent cycles of 1min denaturation at 93°C, 1.5min annealing at 54°C; 2.5min extension at 72°C; and a final extension step of 5 min at 72°C.

The reaction mixture composition was: 10 X PCR buffer (Genedirex USA), 2mM DNTPS, P1+P2 Primers (Sigma Aldrich, United Kingdom), Taq Polymerase (Genedirex USA), Water PCRGrad (Promega). The reaction products were analyzed by agarose gel electrophoresis (2%), and the product was then purified using MiniElute PCR Purification Kit (Qiagen, Germany) and was further subjected to sequencing PCR using the Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystems) and sequenced in the DNA sequencer ABI 310 Genetic Analyzer.

2.6 Rheological properties of fermented dough

The extensibility was tested by Extensograph by adding bacterial isolates to the dough and the results were compared to the control which was fermented using soaked chickpea.

2.7 Statistical analysis

GenState 12.1 was used to analyze the data obtained from dough rising power and Extensograph**.**

Results and Discussions

3.1 Bacterial growth during fermentation

There were three groups of bacteria growing throughout fermentation and also detected at the end of fermentation. One group was cocci which can grow either aerobically or anaerobically and was detected on M17 agar, another one was spore forming bacilli and clostridia which are obligatory anaerobic.

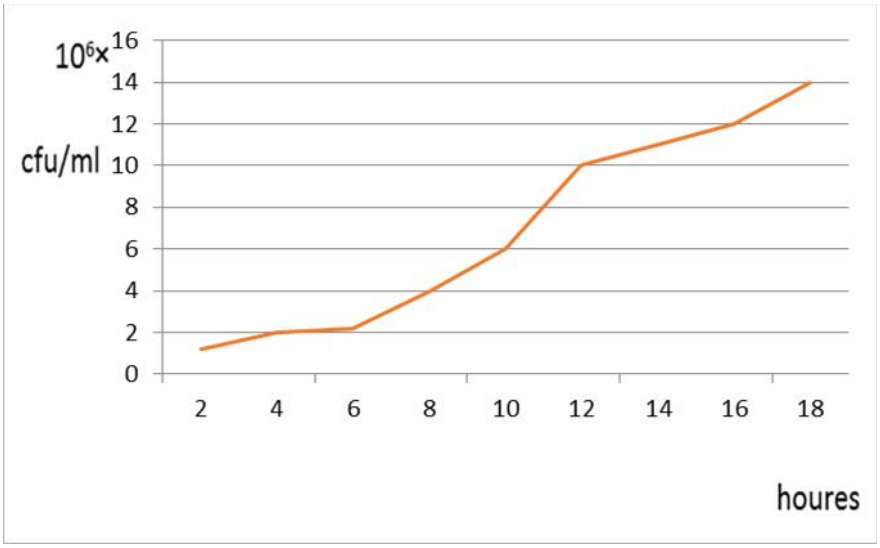

Fig.1: bacteria growth during 18 hours of fermentation on SPS agar

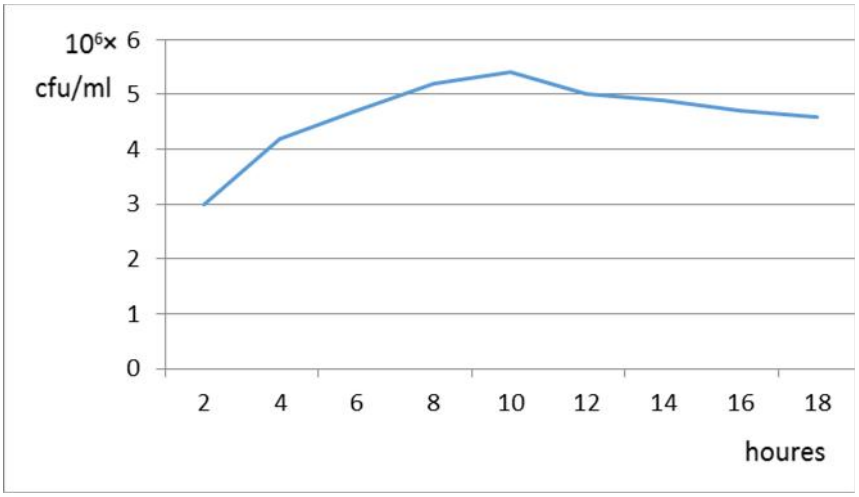

Fig.2: bacteria growth during 18 hours of fermentation on nutrient agar with Ploymyxin

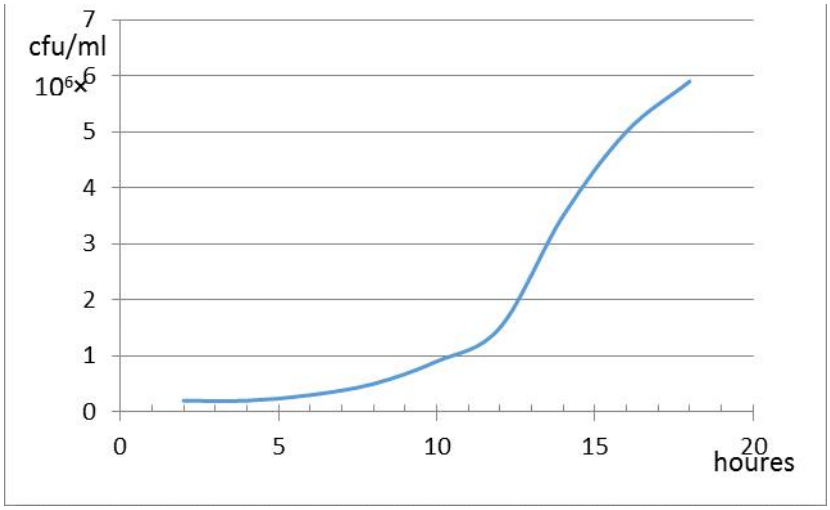

Fig.3: **bacteria growth during 18 hours of fermentation on M17 agar**

The growth curves for different genera isolated during fermentation are shows above (see Fig 1,Fig2,Fig3). Anaerobic bacteria growth was studied during fermentation. The growth was exponentially increased from the beginning of fermentation to reach $14x10^6$ CFU/ml by the end of fermentation (see Fig 2). The number of aerobic spore-forming bacilli increased from the beginning to reach $5.4x10^{10}$ CFU/ml after 10 hours of fermentation and then dropped again to $4.6x10^6$ CFU/ml after 12 hours (see Fig 2). Lactic acid bacteria isolated on M17 agar and clostridia isolated on SPS agar increase started 6-8 hours after soaking and keep increasing to reach $6x10^6$ CFU/ml at the end of fermentation after 12-18 hours (see Fig 1.3).

3.2 pH during fermentation

Results demonstrated that pH was also dropped from 7.5 at the beginning of soaking to 4.2 at the end of fermentation after 12h (see Table 1). The microflora developed during the submerged chickpea fermentation, according to isolation media and microscopic study were *Bacillus* sp. (Cohn 1872), *Clostridium* sp., cocci and lactic acid bacteria.

Table 1: pH changes during chickpea soaking

SOAKING TIME (hr)	2	4	6	8	10	12	14	16	18
pH	7.5	5.5	5.5	5.49	5.49	5.49	5.2	4.44	4.2

3.3 Bacterial identification

During the chickpea fermentation, bacilli lactic acid bacteria were not found according to the biochemical testes shown below (see Tables 2, 3). Anaerobic bacilli were *Clostridium* sp., aerobic bacilli were *Bacillus cereus* (Frankland & Frankland 1887) and coccoid lactic acid bacteria were *Enterococcus* sp [10].All tested isolates did not generate gas from glucose fermentation except *Clostridium* sp. Furthermore, isolates from this genus fermented all tested sugars, except arabinose

and sorbitol, and they also had spores. all isolates can hydrolysis starch, this character important for dough fermentation to get enough glucose for growth , additional test for cocci lactic acid bacteria was done, growth in Bile Escualin medium was positive, and this mean that the isolates was followed *Enterococcus* genus [11], other test for protein crystal was done for aerobic bacilli was positive and this mean it was followed *Bacillus thuringiensis* species [12].

Table 2: Biochemical identification tests for different isolates

TEST		AEROBIC BACILLI		ANAEROBIC BACILLI		COCCI	
Result		**positive**	**negative**	**Positive**	**negative**	**positive**	**negative**
Catalase		10			11		9
Grow at °C	**10**		10		11	9	
	45	10		11		9	
Grow at NaCl %	**4**	10		11		9	
	6.5	10		11		9	
VP		10		11		9	
Starch hydrolysis		10		11		9	
Nitrate reduction		10		11			9
Indol			10		11		9

Table 3: Sugars fermentation for different isolates

	AEROBIC BACILLI	ANAEROBIC BACILLI	COCCI
Glucose	10	11	9
Lactose	0	11	9
Saccharose	8	11	0
Maltose	10	10	9
Arabinose	0	0	9
Rhamanose	0	8	9
Sorbitol	0	0	0
Fructose	10	11	9
Ribose	10	11	9
Xylose	0	8	0

Manittol	0	11	0
Galactose	0	11	9

3.4 DNA purity and concentration

The purity of DNA samples was 1.8-2 and the concentration of the DNA samples were vary from 828.8 in sample 10 to 40 in sample 12 (see Table.4).

Table 4: Purity and concentration of DNA samples

ISOLATE	PURITY $A_{260}/_{280}$	CONCENTRATION A_{260}
1	1.9	161.5
2	2	52.1
3	1.9	209
4	1.9	165.3
5	1.9	155.1
6	2	86
7	2	69.8
8	1.9	152
9	1.9	668.7
10	1.9	828.8
11	1.8	547
12	2	40
13	1.8	483
14	2	93.1
15	1.9	254.7
16	1.8	688.6
17	1.9	649.4

18	1.9	184.6
19	2	193.1
20	2	128.3
21	1.9	121.7
22	2	76.4
23	1.9	470
24	1.9	72
25	1.8	354.1
26	2	361.9
27	2	101.5
28	1.9	356.8
29	1.9	140.9
30	1.9	282.3

3.5 16S rDNA analysis

Most studied isolates gave one band between 1100-1050 bp using universal primers P1/P2 (see Figure 2). DNA from one representative strain of each group/subgroup was extracted and 16S rDNA gene fragments were amplified and sequenced. GenBank database [13] was used to compare 16S rDNA sequences and three species were identified as *E. faecium*, *B. thuringiensis* and *Clostridium sartagoforme*..

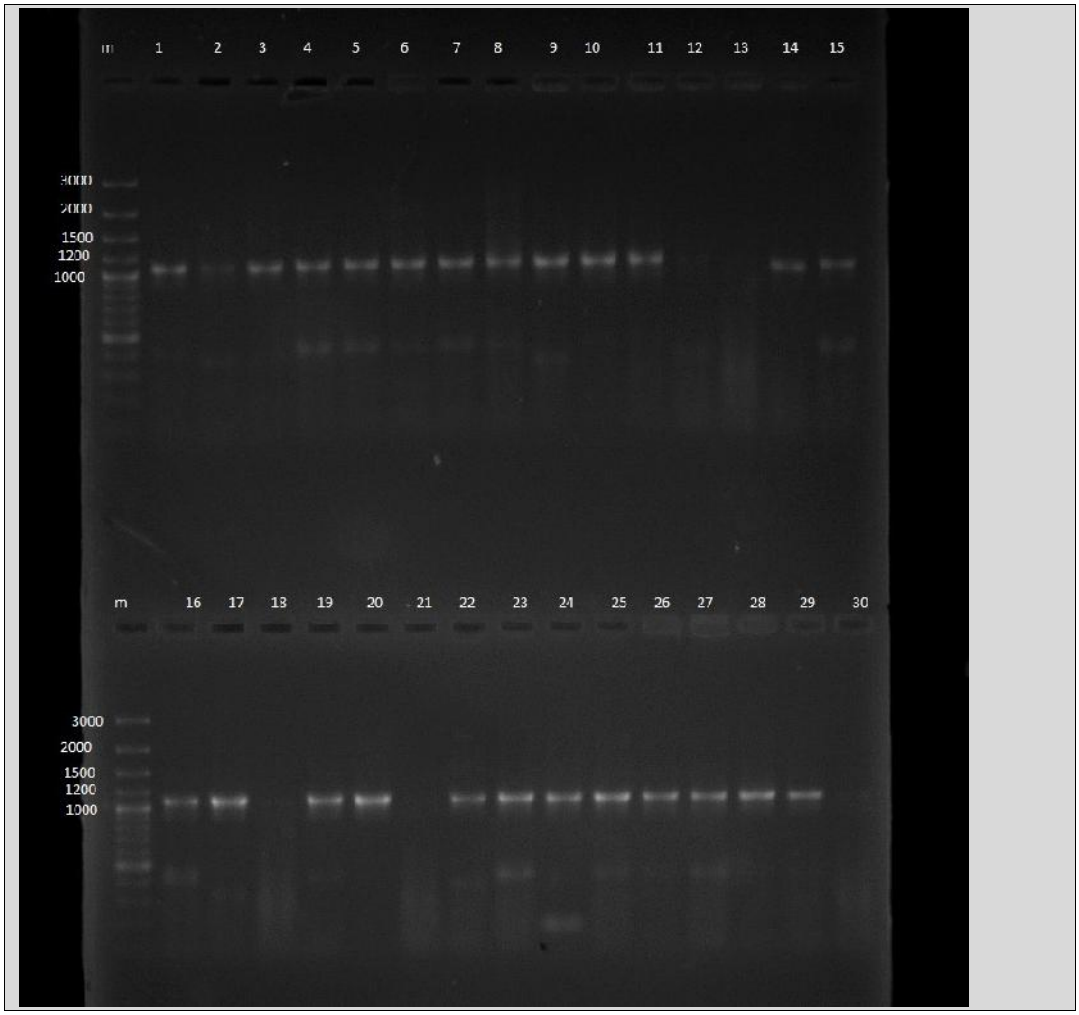

Fig .4: Agarose gel for PCR products from different strains isolated during fermentation

Table 5: Values of parameter given by Extensograph

PARAMETER ISOLATE	E mm	R5 BU	Energy Cm
Control	107.5a	325d	60.8e
Clostridium	95b	310d	46ef

Enterococcus	72.5c	310d	57.4ef
Bacillus	70c	300d	35.6f

*Value of L.S.D at 1% level for E = 11.71;
** Value of L.S.D at 1% level for R5 = 85.37;
*** Value of L.S.D at 1% level for Energy = 38.38.
The different letter means a significant difference in each column, at energy column the shared letter means there is no significant difference between isolates.

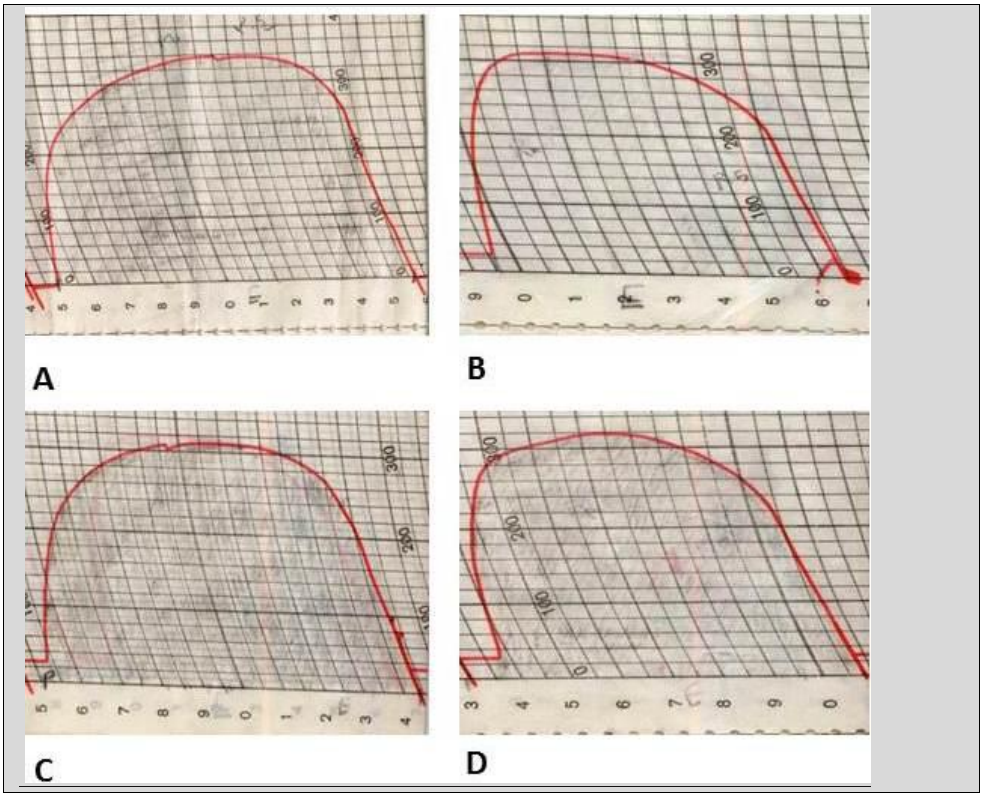

Fig .5: Extensograph plots of the three isolates and control. (a) Dough fermented by soaked fermented chickpea (control); (b) Dough fermented by *Bacillus*. isolate; (c) Dough fermented by *Clostridium*. isolate; (d) Dough fermented by *Enterococcus*. isolate (original)

3.6 Rheological study

The absorbed water for flour by farinograph was 58%; each isolate of bacteria was tested and used to ferment dough by Extensograph for 75 minutes. Extensograph plots of the three isolates and control were shown above (see Fig 5). The calculated parameters shows that the highest extensibility (E) after control was for dough fermented by *Clostridium*. isolate (see Table 5). Results showed that the biggest increase in the dough volume was for dough fermented by *Clostridium.*

although it took more time to reach this increase than the control. Both doughs prepared with *Clostridium* sp. and control decreased in volume after 6 hours (see Table 6).

Table 6: Dough rising power for the isolated strains compared to chickpea soak (control)

ISOLATE TIME (hr)	Control	Btb	Cla	E
2	100%a	7%	50%	7%
4	68%	7%	110%a	7%
6	60%	7b%	62%	10%b

*Different letters means there is a significant difference between values

The pH drop during the first 10 hours of fermentation (see Table 1) was mainly due to the activity of the continuously increasing population of bacilli [14]

It was interesting to see that no microbial groups other than bacilli, clostridia and coccoid lactic acid bacteria were detected during the fermentation. Biochemical identification (see Tables 2, 3) and rDNA analysis confirmed that only these groups are present in chickpea soak.

The rheological study demonstrated that Clostridium. isolates showed the highest extensibility (E) and dough fermentation. This was consistent with the biochemical testes which showed that all tested isolates did not generate gas from glucose fermentation except Clostridium.

Conclusions

The major bacteria genera in the soaked fermented chickpea were *Clostridium* sp., *Bacillus* sp. and *Enterococcus* sp. but only *Clostridium* sp. produced gas from sugar and hydrolysis gluten during dough fermentation which gives Kaak it is special texture.

These results suggested the possibility of using *Clostridium sartagoforme*. isolated from chickpea soak for production of this famous traditional pastry in Syria and Middle East to avoid the problems of the very short shelf life of the soaked chickpea and instability of its properties according to the success of soaking process.

This work will require optimization of the starter bacteria addition amounts and sensory tests for the product in order to get same traditional taste of Kaak.

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