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Microbial composition of kefir produced by a novel method in Syria

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Abstract : Kefir is one of the important fermented milk product which have many beneficial health effects. However, The method of their preparation was handed down as a precious inheritance from father to son. So we can say that the production of kefir grains is still a mysterious secret. In the current study we developed a very easy method for production of kefir grains in short time (72 h.), moreover the microbial composition of the resultant kefir grains was unique and different from the other products found in other countries.
The microbial composition of kefir grains were investigated using biochemical tests of three commercial systems: API 50 CHL for identification lactic acid bacteria belonging to the genera *Lactobacillus* and *Lueconostoc*; ID 32 STREP for identification of the genera *Lactococcus*, *Streptococcus* and *Enterococcus*; while yeasts were identified using ID 32 C system.
The biochemical tests of the commercial systems revealed the existence of 17 species bacteria, and 4 species of yeasts. The species/subspecies bacteria were: *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus curvatus* ssp. *curvatus*, *Lactobacillus delbrueckii*ssp. *bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus paracasei* ssp. *paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus* pentoses, *Leuconostoclactis*, *Leuconostoc mesentroides* ssp. *cremoris*, *Leuconostoc mesentroides* ssp. *dextranicum*, *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus raffinolactis*, *Streptococcus thermophiles* and *Enterococcus durans*. While the yeasts species were: *Saccharomyces cervisiae*, *Saccharomyces kluverii*, *Kluyveromyces marxiamus*, *Zygosaccharomyces* spp.
Keywords : Kefir; API 50 CHL; ID 32 STREP; ID 32 C; *Lactobacillus*; *Leuconostoc*; *Streptococcus*; *Lactococcus*; *Enterococcus*; yeasts.

Introduction

Production of the first fermented milks dates back to 7000 BC with origins in the middle and far-east of Asia, making it one of the oldest methods of long term food preservation. A further spreading east of these traditions, by way of Russia and Eastern Europe, by the Tartars, Mongols and Huns occurred during their conquests[1].

Fermentations in milk occur as a result of the action of lactic acid bacteria, and occasionally, lactose fermenting yeasts, on lactose. Lactic acid bacteria prefer lactose as their source of carbon, and the end products can be exclusively lactic acid, or other substances may be produced, such as acetic acid, carbon dioxide and hydrogen[2]. Yeasts, such as *Saccharomyces cerevisiae*, are also capable of fermenting lactose and other sugars and can be found in some fermented milks[3].

Kefir is one of the important fermented milk product which was originated in central Asia between the Caucasus Mountains and Mongolia, and is very popular in many countries nowadays, such as Turkey, Russia, Poland, Czech Republic, Slovakia, Hungary, Bulgaria, Scandinavian countries, The United States, Brazil and Japan [4,5,6,7,8,9,10]. It is a carbonated fermented milk product made by using a complex mixture of microorganisms bound together in an exopolysaccharide matrix produced by certain lactic acid bacteria known as kefir grains[11].

Kefir is characterized with a smooth and creamy texture and has an acidic and slightly alcoholic and yeasty taste; the presence of carbon dioxide gives a varying degree of effervescence[12]. In fact, the word kefir is derived from the Turkish word 'kef', which means pleasant taste[13].

Commercial kefir is produced by using a pure starter culture consisting of a mixture of bacteria and yeast species that give a flavor similar to traditional kefir, but some health benefits may be lost in commercial production due to reduced microbial diversity and lack of beneficial exopolysaccharides[2]. The starter cultures, termed "grains", grow, propagate and pass their properties along to the following generations of grains[14].

Health aspects attributed to the consumption of kefir, as similar to other fermented dairy foods supplemented with probiotic bacteria, include, but are not limited to, improved lactose utilization, anticarcinogenic activity, control of intestinal infections and improved flavor and nutritional quality of the milk[15].

A very complicate, time consuming method to produce kefir grains was described by traditional method[16], since kefir grainscannot be synthesized artificially. Kefir grains do not form spontaneously when pure cultures of the organisms involved are placed together in a test tube. But under the proper conditions, they can apparently be encouraged to form and grow in traditional ways. The traditional way to ferment milk for kefir was in goatskin bags. Fermentation of milk in skin bags as a way of preserving the milk led to the first kefir grains and started the long tradition of producing kefir [16]. These bags would traditionally be hung by entrances to peoples' homes, where people entering or leaving would kick or hit the bags to agitate the contents[17]. Bags could also be carried as people traveled, the bumpiness of the ride mixing the contents. Motaghi *et al.*[18] tested this hypothesis with some success when they filled a goat-hide bag with pasteurized milk and intestinal flora from sheep, incubating at 24–26°C, shaking hourly, and replacing 75 % of the milk at a time as it coagulated. After 12 weeks, a polysaccharide layer had formed on the surface of the hide. This was removed and propagated in milk. From this, they were apparently able to obtain kefir grains.

Materials and Methods

Goat colostrum was obtained from Damascus, Syria. Natural microflora was used to ferment colostrum to produce kefir grains, by incubating 500 ml of fresh colostrum at 37° C (Memmert Co., Germany) for 72 h, with stirring occasionally (every 4 h.). This process was repeated 3 times to ensure the constant properties of kefir grains produced. Kefir grains were obtained by filtration of colostrum including kefir grains by means of clean gauze.

Resulted kefir grains were investigated to identify the microbial composition. They were divided into small pieces using sterilized knife, which in turn were used to prepare serial dilutions in sterilized saline (0.85% sodium chloride), ranged from 10^{-1} to 10^{-9}. 1 ml of each dilution was plated in three types of media, namely: MRS agar, to isolate bacilli lactic acid bacteria; M7 agar to isolate cocci lactic acid bacteria; malt extract agar to isolate yeasts.

Well isolated colonies were streaked on agar plates (the same type of the original plates); to ensure obtaining pure cultures, this step was repeated twice.

Pure cultures of bacilli and cocci lactic acid bacteria were tested for Gram reaction, catalase production. Then bacilli lactic acid bacteria were identified using API 50 CHL system, while cocci lactic acid bacteria were identified by means of ID 32 STREP system, and yeasts using ID 32 C system; according to manufacturer instructions. The results of biochemical tests were interpreted by means of abi*web* V 4.1 software (bioMérieux, Marcy-l'Etoile, France).

Results and Discussion

Results of identification studies showed the unique lactic acid bacteria and yeast profiles of the novel kefir grains produced in Syria. The current study reveals the existence of 17 species of lactic acid bacteria, and 4 species of yeasts. Lactic acid bacteria species/subspecies were as follows:9 bacilli species/subspecies, namely: *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus curvatus* ssp. *curvatus*, *Lactobacillus delbrueckii*ssp. *bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus paracasei* ssp. *paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus pentoses*; and 8cocci species/subspecies, namely: *Leuconostoc lactis*, *Leuconostoc mesentroides* ssp. *cremoris*, *Leuconostoc mesentroides* ssp. *dextranicum*, *Lactococcuslactis* ssp. *lactis*, *Lactococcuslactis* ssp. *cremoris*, *Lactococcus raffinolactis*, *Streptococcus thermophiles* and *Enterococcus durans*. While the yeasts species were: *Saccharomyces cervisiae*, *Saccharomyces kluverii*, *Kluyveromyces marxiamus*, *Zygosaccharomyces* spp. Table 1. illustrates the results of biochemical tests of API 50 CHL system, and Table 2.shows them using ID 32 STREP system. The two systems were used to identify lactic acid bacteria. While Table 3. revealed the biochemical tests of ID 32 C system used to identify yeasts.

Table 1.The results of biochemical tests for identification of lactic acid bacteria using API 50 CHL system.

Test	*Lb.paracasi*	*Lb. rhamnosus*	*Lb. brevis*	*Lb. plantarum*	*Lb.fermentum*	*Lb. pentoses*	*Lactobacillus acidophilus*	*Lb.curvatus*ssp.*curvatus*	*Lb.delbrueckii*ssp. *bulgaricus*	*Leuconostoc mesentroides*ssp.*cremoris*	*Leuconostoclactis*	*Leuconostocmesentroides* ssp.*dextranicum*
0	−	−	−	−	−	−	−	−	−	−	−	−
GLY	−	−	−	−	−	+	−	−	−	−	−	−
ERY	−	−	−	−	−	−	−	−	−	−	−	−
DARA	−	−	−	−	−	−	−	−	−	−	−	−
LARA	−	−	+	+	−	+	−	−	−	−	−	+
RIB	+	+	+	+	+	+	−	+	−	−	−	−
DXYL	−	−	+	−	+	+	−	−	−	−	−	+
LXYL	−	−	−	−	−	−	−	−	−	−	−	−
ADO	−	−	−	−	−	−	−	−	−	−	−	−
MDX	−	−	−	−	−	−	−	−	−	−	−	−
GAL	+	+	+	+	+	+	+	+	−	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+	+	+
FRU	+	+	+	+	+	+	+	+	+	−	+	+
MNE	+	+	+	+	+	+	+	+	−	−	+	+
SBE	+	+	−	−	−	−	−	−	−	−	−	−
RHA	−	+	−	−	−	−	−	−	−	−	−	−
DUL	−	−	−	−	−	−	−	−	−	−	−	−
INO	−	−	−	−	−	−	−	−	−	−	−	−
MAN	+	+	−	+	−	+	−	−	−	−	−	−
SOR	+	+	−	+	−	+	−	−	−	−	−	−
MDM	−	−	−	+	−	−	−	−	−	−	−	−
MDG	−	+	−	−	−	+	−	−	−	−	−	+
NAG	+	+	+	+	−	+	+	+	−	+	+	+
AMY	+	+	+	+	−	+	+	−	−	−	−	+
ARB	+	+	+	+	−	+	+	−	−	−	−	+

ESC	+	+	+	+	−	+	+	−	−	−	−	+
SAL	+	+	+	+	−	+	+	−	−	−	−	+
CEL	+	+	+	+	−	+	+	−	−	−	−	+
MAL	+	+	+	+	+	+	+	+	−	−	+	+
LAC	+	+	+	+	+	+	+	+	+	−	+	−
MEL	−	+	+	+	+	+	−	−	−	−	+	+
SAC	+	+	+	+	+	+	+	−	−	−	+	+
TRE	+	+	+	+	−	+	+	−	−	−	−	+
INU	−	−	−	−	−	−	−	−	−	−	−	−
MLZ	+	+	−	+	−	−	−	−	−	−	−	−
RAF	−	−	−	+	−	+	−	−	−	−	−	−
AMD	−	−	−	−	−	−	−	−	−	−	−	−
GLYG	−	−	−	−	−	−	−	−	−	−	−	−
XLT	−	−	−	−	−	−	−	−	−	−	−	−
GEN	+	+	+	+	−	+	+	−	−	−	−	+
TUR	+	+	+	+	−	+	−	−	−	−	−	+
LYX	−	−	−	−	−	−	−	−	−	−	−	−
TAG	+	+	−	−	−	−	−	−	−	−	−	−
DFUC	−	−	−	−	−	−	−	−	−	−	−	−
LFUC	−	−	−	−	−	−	−	−	−	−	−	−
DARL	−	−	−	−	−	−	−	−	−	−	−	−
LARL	−	−	−	−	−	−	−	−	−	−	−	−
GNT	+	+	+	+	+	+	−	−	−	−	−	−
2KG	−	−	−	−	−	−	−	−	−	−	−	−
5KG	−	−	−	−	−	−	−	−	−	−	−	−

Table 1. which illustrates the results of biochemical tests in API 50 CHL system used to identification of lactic acid bacteria belonging to the genera *Lactobacillus* and *Leuconostoc,* revealed that all species/subspecies were ERY, DARA, LXYL, ADO, MDX, DUL, INO, INU, AMD, GLYG, XLT, LYX, DFUC, LFUC, DARL, LARL, 2KG and 5KG negative; while all of them were GLU positive. *Lactobacillus rhamnosus* the only species RHA positive, and *Lactobacillus plantarum* was the only one MDM positive, while *Lactobacillus pentoses* was unique in its ability to ferment GLY. All species/subspecies were able to ferment GAL except for *Lactobacillus delbrueckii* ssp. *bulgaricus*, while all of they could ferment FRU except for *Leuconostoc mesentroides* ssp.*cremoris*.

Referring to table 2. which revealed the biochemical tests used to identify lactic acid bacteria that belonging to the genera *Lactococcus*, *Streptococcus* and *Enterococcus* it can be noticed that all species/subspecies were βGUR, SOR, LARA, DARL, GTA, GLYG, PUL, MEL, MLZ and TAG negative; while all of them were LAC and VP positive. *Lactococcuslactis* ssp.*cremoris* was the only species/subspecies who was HIP positive; while *Lactococcus raffinolactis* the only one αGAL, PAL, MAN, RAF positive and *Streptococcus thermophiles* characterized in positive tests of βGAR, SAC, URE; while *Enterococcus durans* characterized in positive PYRA and negative APPA tests.

Table 3. which concern in biochemical tests of yeasts isolated from locally produced kefir grains, shows that all species were NAG, CEL, INO, XYL, RIB, RHA, ERY, GRT, MLZ, GNT, LVT, SBE, GLN negative and GLU positive. *Saccharomyces cerevisiae* was unique in its ability to ferment LAC, while *Saccharomyces kluverii* was the only species positive for TRE, 2KG, MDG, PLE and MEL. *Kluyveromyces marxiams* characterized in positive ACT and ARA, and negative MAN tests; while *Zygosaccharomyces* sp. was the only species who could grow in presence of GLY as a sole for carbon, and was GAL, SAC, LAT and RAF negative.

Table 2.The results of biochemical tests for identification of lactic acid bacteria using ID 32 STREP system.

Test	Lactococcus lactis ssp.cremoris	Lactococcuslactisssp. lactis	Lactococcus raffinolactis	Streptococcus thermophiles	Enterococcus durans
ADH	−	+	−	−	+
βGLU	−	+	+	−	+
βGAR	−	−	−	+	−
βGUR	−	−	−	−	−
αGAL	−	−	+	−	−
PAL	−	−	+	−	−
RIB	−	+	−	−	+
MAN	−	−	+	−	−
SOR	−	−	−	−	−
LAC	+	+	+	+	+
TRE	−	+	+	−	−
RAF	−	−	+	−	−
SAC	−	−	−	+	−
LARA	−	−	−	−	−
DARL	−	−	−	−	−
CDEX	−	+	+	−	+
VP	+	+	+	+	+
APPA	+	+	+	+	−
βGAL	−	−	−	+	+
PYRA	−	−	−	−	+
βNAG	−	+	+	−	+
GTA	−	−	−	−	−
HIP	+	−	−	−	−
GLYG	−	−	−	−	−
PUL	−	−	−	−	−
MAL	−	+	+	−	+
MEL	−	−	−	−	−
MLZ	−	−	−	−	−
MβDG	−	+	−	−	+
TAG	−	−	−	−	−
βMAN	−	+	−	−	+
URE	−	−	−	+	−

Table 3.The results of biochemical tests for identification of yeasts using ID 32 C system.

Test	*Saccharomyces cerevisiae*	*Saccharomyces kluverii*	*Kluyveromyces marxiams*	*Zygosaccharomyces* sp.
GAL	+	+	+	−
ACT	−	−	+	−
SAC	+	+	+	−
NAG	−	−	−	−
LAT	+	+	+	−
ARA	−	−	+	−
CEL	−	−	−	−
RAF	+	+	+	−
MAL	+	+	−	−
TRE	−	+	−	−
2KG	−	+	−	−
MDG	−	+	−	−
MAN	+	+	−	+
LAC	+	−	−	−
INO	−	−	−	−
SOR	−	+	−	+
XYL	−	−	−	−
RIB	−	−	−	−
GLY	−	−	−	+
RHA	−	−	−	−
PLE	−	+	−	−
ERY	−	−	−	−
MEL	−	+	−	−
GRT	−	−	−	−
MLZ	−	−	−	−
GNT	−	−	−	−
LVT	−	−	−	−
GLU	+	+	+	+
SBE	−	−	−	−
GLN	−	−	−	−
ESC	−	−	+	−

In the current study, the microbial composition of locally produced kefir by a novel method was investigated. The biochemical tests carried out to identify lactic acid bacteria using the API 50 CHL and ID 32 STREP system revealed the existence of 17 species/subspecies: 9 *Lactobacillus*, 3 *Lueconostoc*, 3 *Lactococcusi*, 1 *Enterococcus* and 1 *Streptococcus* species/subspecies. These results agree with that recorded by Magalhães*et al*.[19]in their study on Brazilian kefir, who found that Lactobacilli were the predominant lactic acid bacteria type, with Lactococci comprising the majority of the remaining. However the of *Lactobacillus* species/subspecies were different in number and type: in current study there were 9 species/subspecies: *Lactobacillus acidophilus*, *Lactobacillus brevis*- a kefiran producer[20]-*Lactobacillus curvatus* ssp. *curvatus*, *Lactobacillus delbrueckii*ssp. *bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus paracasei* ssp. *paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus pentoses*; while Magalhães*et al*.[19] found only 4 species: *Lactobacillus paracasei*, *Lactobacillus parabuchneri*, *Lactobacilli casei* and *Lactobacilli kefiri*. The existence of *Lactobacillus plantarum*in kefir grains was recorded by Golowczyc*et al*.[21], who found that it was predominant species of *Lactobacillus* bacteria. However, Leite*et al*.[22] andKök*et al*.[23]recorded that *Lactobacillus acidophilus* is one of the predominant *Lactobacillus* species found in many kefir grains, and Singh *et al*.[24]and Delfederico*et al*.[25]revealed that *Lactobacillus delbrueckii ssp. bulgaricus* and *L. fermentum, L. rhamnosus* was found in kefir grains.

The current study revealed the existence of 3 species/subspecies belonging to the genus *Lactoccus*: *L. lactis*ssp. *lactis*, *L. lactis*ssp. *cremoris* and *L. raffinolactis*. This result partially agreed with that recorded by Magalhães*et al*. [19], who found that the only *Lactococcus* species could be isolated from Brazilian kefir was *L. lactis*.

The only species of yeasts found to be able to ferment lactose in the current study was *Saccharomyces cerevisiae*, which enhance the sensory qualities of the kefir beverage, as it promotes a strong and typically yeasty aroma, as well as a refreshing taste[19]. The other two non-lactose fermenting species were *Saccharomyces kluverii*, *Kluyveromyces marxiams* and *Zygosaccharomyces* sp.; the last result disagreed with Magalhães, *et al*.[19], who found the non-lactose fermenting yeast in Brazilian kefir were *Kazachstania aerobia* and *Lachancea meyersii*, this may due to the differences in methods in which the two types of kefir (Brazilian and Syrian) were prepared, and the differences in natural microflora in the raw materials. Previous researches revealed the major yeast in kefir grains are *Kluyveromyces maxianus*, *Torulaspora delbrueckii*, *Saccharomyces cerevisiae*, *Candida kefir*, *Saccharomyces unisporus*, *Pichiafermentans*, *Kazachastania aerobia*, *Lachancea emeyersii*, *Yarrowia lipolytica*, and *Kazachstaniaunispora*[14,26,27,28].

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