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Chemical and microbiological composition of Kefir and its natural benefits

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Abstract: Kefir grains or Tibetan mushrooms are considered a natural remedy thanks to their various curative properties against chronic diseases and certain cancers. They represent a symbiotic consortium of yeast and bacteria with high similarity to those present in the intestine. For this reason, the consumption reinforces the intestinal flora, helps with digestion, restores the digestive system and strongly stimulates the immune system.

In association with milk and slightly thickened with yogurt, kefir has the benefits of a pro-biotic since it is rich in vitamins, minerals and polysaccharides.

In this study we identified the diversity of bacteria, yeast and mould contained in kefir, a diversity which is important for its curative role in many disorders like diabetes and cancer. In a future study, we aim to investigate the mechanisms by which Kefiran; the main effective agent; regulates cell growth and immunity.

Keywords: Kefir, grains, mould, bacteria, yeast, Kefiran

Introduction

The word kefir comes from the Caucasus language indicating a fermented drink made of milk. It is thought that kefir appeared when the nomads transported reindeer, goat, ewe, cattle, camels or other animals' milk in bags of skin. This resulted in the fermentation and the formation of a beverage, with a characteristic taste and a better conservation [1]. Kefir was brought for the first time by a polish professor to a private clinic in Gliwice, (in Silesia, close to the Czech Republic). He got sick during his five years stay in India, suffering from liver cancer. Completely cured with this mushroom by Indian monk, he decided to take some with him back to his country.

He started to distribute the grains to make profit of its knowledge and to supply patients in need. Kefir first became very popular in Poland than in all over Europe.

In 1889; Beijerinck a microbiologist of the XIX century who was interested in spontaneous fermentations, said: "By kefir one understands the leaven of milk of the mountain tribes of the Caucasus, and also the drink resulting from its action on milk. The true name of this drink, however, is "sakwaska". From here on, we will indicate the leaven under the name of "grains of Kefir" [2].

The composition of grains of Kefir is 90% water, 10% dry matter, the last is made of 3-4% fats, 30% proteins, 7% ash and 55% of non-nitrogenous extractable substances NES, see table.1[3]. Almost 50% of polysaccharides, accounting for 24% of the dry weight of the grains of kefir can be precipitated by the addition of alcohol [4]. Hydrolysis of the polysaccharides produced only D-glucose and D-galactose in the proportion of 1:1. This polysaccharide was found only in kefir and is named kefiran.

Table 1: Chemical composition of Kefir seed from different countries

Country of origine	water %	Dry matter %	Dry matter composition			
			Fat %	Proteins %	NES* %	ash %
Russia	89,5	10,5	2,8	30,3	59,3	7,6
Yugoslavia	88,9	11,1	4,3	31,4	57,2	7,2
Bulgaria	90,6	9,4	3,5	34,4	53,4	8,7

*Non-nitrogenous extractable substances

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The molecular structure of Kefiran is still not entirely known. It is thought to be composed of a repeated moiety of ramified hexa or hepta saccharide (Figure 1). The moiety itself is made of a penta saccharide with one or two sugar residues bound randomly. The variety of bounds makes kefiran resistant to digestion enzyme attack [5]. This propriety plays an important role in the ecological stability and the therapeutic activity of Kefir grains. Thus, kefiran seems reasonably inert and fully resistant to digestive enzymes.

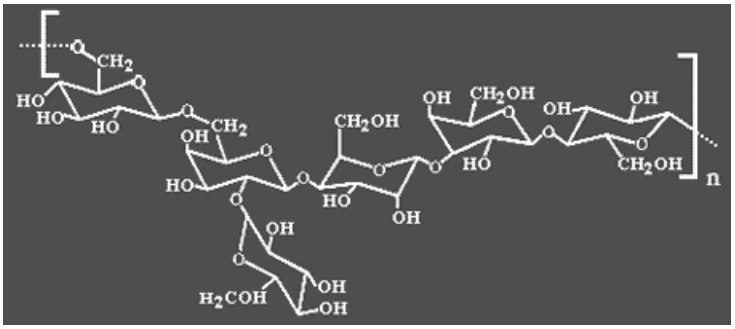


Figure 1: **The proposed molecular structure of Kefiran**

The grains of kefir are gelatinous and groove-like particles resembling to small balls that develop in fermented milk containing lactic acid bacteria and yeasts capable of fermenting lactose. They are insoluble in water and in most solvents. In a fresh state, they are white while in the dry state, the masses are hard and yellow containing microorganisms which vitality depends on the conditions of drying. In milk, the dry grains inflate and blanch [6]. See Figure 2.

Figure 2: **Kefir grains**

Several researchers have described the grains of kefir as being "small wrinkled masses up to the size of a walnut with a cauliflower shape", but sometimes they can unroll into sheet" (Figure 3) [7,8].

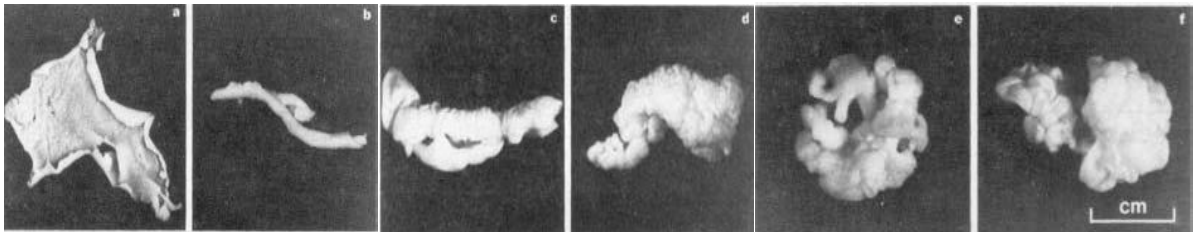


Figure 3: **Sheets of Kefir grains- a** : Sheet ; **b** : enrolled sheet ; **c** andt **d** : undefined shape ; **e** and **f** : cauliflower shape

These grains may aggregate to form microbial structures like soft biofilms floating in milk. The polysaccharides in Kefir grains were analyzed and found to be all dextranes with a principal chain of (1-6)-α-D-glucose bound on C3 like those produced by *Lactobacillus brevis* and *Leuconostoc mesenteroides*. Those microorganisms may have a direct important role in the grains formation together with other yeast and *Lactobacillus casei* which can facilitate the dextrane condensation [8].

Fats, lactic acid and alcohol are the most important components. Fats depend on milk origin (cow, sheep, goat) and type (whole, skimmed or half skimmed). See Table 2 for the change in kefir composition over time of fermentation.

Table 2: **The composition of kefir over time according to Bogolubo**.

Time of fermentation	24 h	48h	72 h
	%	%	%
Fats	3,62	3,63	3,63
Proteins	3,06	3,08	3,07
Lactose	2,78	2,24	1,67
Lactic acid	0,76	0,83	0,90
Alcohol	0,63	0,81	1,10
Water and minerals	89,15	89,41	89,63
	100,00	100,00	100,00

The rates of lactic acid concentration varies between 0.6 and 0.9%, the alcohol level between 0,01% and 1% [9-11]. Kefir commercially produced in Germany contained less than 0,01% alcohol [6,12]. The composition of kefir reflects the difference in commercial processes, an optimal proportion of 3:1 between the di-acetyl and the acetaldehyde is needed to provide the typical flavor of kefir [11]. Propionaldehyde, methyl ethyl ketone-2, N-propanol, isoamyl alcohol and the acetic acid are also important for the kefir flavor but vary considerably during maturation of the product.

Material and methods and results:

Sample preparation:

For the preparation of the samples, we put initially 10 g of grains in a jar, then we add milk, leaving a margin because fermentation makes the volume inflates . We pose a sheet of absorbing paper retained by a rubber band or a compress over the jar. The container is to be placed safe from direct sunlight. We left the compound to mature at room temperature, until the desired ripening that varies from 12 h to 24 h according to the temperature and the volume of milk desired acidity. As soon as kefir is ready, we pass it through a plastic strainer and then store until use.

Physicochemical characters

Density:

We were interested in this part to the study in the density of the samples of kefir. This study was made on 2 samples of grains originated from Turkey but of two different sources sample 1 symbolized as P (with cauliflower shape) and the second as H (sheet).
The tests carried out on kefir grains were compared to pilot skimmed milk symbolized by an R; used as a control.

Density is determined using a lacto-densitometer. The prepared samples have curdled milk which means their density is higher than normality (1.023 to 1.035)

Acidity:

Milk acidity is due to lactic acid resulting from the fermentation of glucose and galactose both products of lactose hydrolysis in milk. Milk acidity can be titrated directly by soda in the presence of phenolphthalein.

The proportion of lactic acid is indicator of samples acidity.
Acidity of sample P= 165°Dornic Acidity of sample H= 170°Dornic
A light increase in acidity of sample H compared to Sample P at 24 h

For a better evaluation of the acidity of the 2 samples and the pilot milk, measurements were carried out till 48h see Table 3.

Table 3: Evaluation of Kefir acidity at 48h

Acidity	0h	2h	4h	6h	8h	24h	48h
P °D	16	26	34	38	46	165	162
H°D	16	26	35	40	50	170	166
R°D	16	16	16	18	18	66	70

Measurement of fats:

We also were interested in the investigation of fat content, the lipid and lipoid compounds of milk were determined by the method GERBER (acid-butyric- metric). After dissolution of proteins by addition of sulfuric ml of acid H_2SO_4, the separation of milk fats performed by centrifugation in a butyric-meter is supported by the addition of 1 ml of Iso-amyl alcohol $C_5H_{12}O$.
The calculation of milk fats is done using the following formula:

Fat (g/l) = (B-A)

Where A: the value corresponding to the lower level of the fatty column. B: the value corresponding to the higher level of the fatty column.
We obtained the following results:

Fat. P= 8g/l Fat .H= 9g/l

Note: basic milk used is skimmed milk and does not contain fat.

The calculation of the total dry extract (TDE) is the difference in weight before and after the desiccation in a dry oven.

TDE g/l= (p2-p1) x 100%
TDE . P= (25,7-25) x100% = 70%
TDE .H= (22,6-21,8) x100% = 80%

pH kefir:

The measurement of pH gives an idea on the microbiological composition of the samples especially the pathogenic germs. The results are represented in table 4. A rating curve of pH during 24h is plotted on Figure 4.

Table 4: Evaluation du pH durant la fermentation dans les 24h

	0h	2h	4h	6h	8h	10h	12h	24h
pH.P	6,6	5,38	3,86	3,75	3,61	3,50	3,43	3,40
pH.H	6,6	5,30	3,63	3,47	3,42	3,34	3,29	3,27

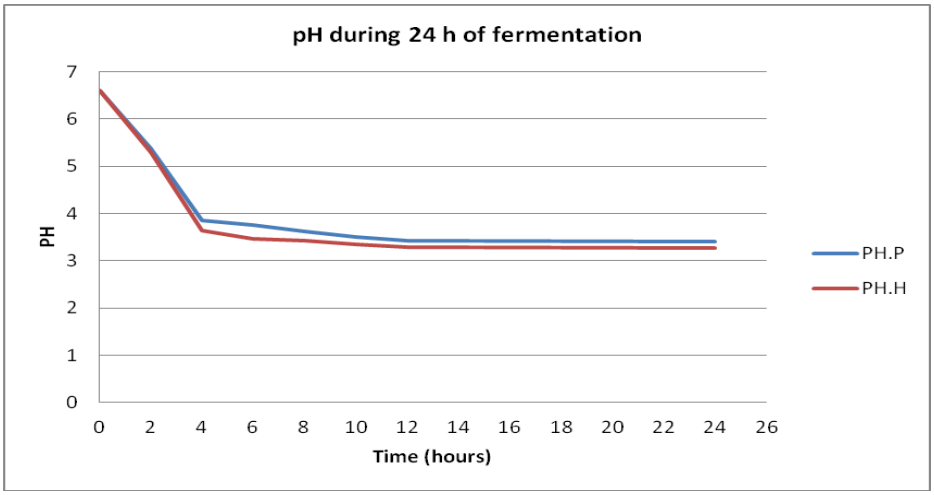


Figure 4: **pH measurements during 24 hours of kefir fermentation**

Microbiological study:

Mesophilic aerobic bacteria

The culture is done on plate count agar (PCA) medium, a microbiological growth medium commonly used to assess and monitor "total" or viable bacterial growth of a sample. PCA is not a selective medium and its composition may vary, but typically it contains 0.5% peptone. The culture was kept during 48h at 37°C, the reading and counting are performed by a germ-meter COLONYSTARGMBH 8501. Only eye-shaped colonies will be counted. The number found will be multiplied by the reverse of dilution factor (3) which means $x10^3$
(P) = $43x10^3$ CFU/ml (H) = $35x10^3$ CFU/ml

Coliforms:

When using the Violet Red Bile Glucose (VRBG) medium, for the detection and enumeration of Enterobacteriaceae in our samples, no colonies have grown at 48 h.

With Shigella salmonella medium, normally the colonies are transparent with black centre, but in our case no colonies were revealed.

Clostridium:

The presence of black spots and a release of gas normally indicate clostridium colonization. Our samples do not present colonies of clostridium since no change of agar was observed on the sample tubes in Figure 5.

Figure 5: Tube incubation for clostridium colonization

Enterococci (streptococcus):

No changes were observed in the tubes with Evalitsky culture meaning the samples do not contain streptococci as shown in Figure 6.

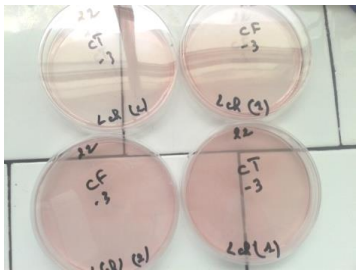


Figure 6: Tube incubation for streptococci colonization

Figure 7: Plate incubation for staphylococci colonization

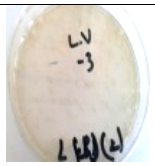
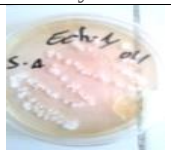
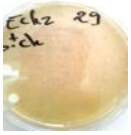
Staphylococci:

If the Giolitti cantoni broth, the color turns to black, the presence of staphylococci needs to be confirmed by plating the broth on chapemann medium. The medium will turn from red to yellow in the presence of staph which was the case for sample H (Figure 7).

Yeast and moulds

Sabouraud medium is a selective medium for yeasts and moulds, the colonies are round and creamy with some mould fluffy colonies (see Table 5)

Table 5: Incubations in sabouraud medium of samples P and H for 72h

	Sample P	Sample H
Normal Sabouraut	 Creamy colonies + mould	 Creamy colonies + fluffy mould
Sabouraut + actidion	 Fluffy white colonies	 Fluffy white inflated colonies
Sabouraut + chloramphenicol	 Fluffy white lobed colonies	 Fluffy white lobed colonies

The counting of colonies by the previous methods and tests are represented in Table 6:

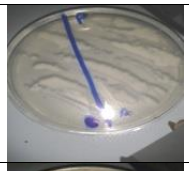
Table 6: Counting colonies

		FMAT	CT	CF	Staph	Clost	S.S	Strep	yeast	Mould
P	Kefir	$43x10^3$	abs	abs	10	abs	abs	abs	$47x10^3$	10^3
	grains	190x10	abs	abs	10	abs	abs	Pres.	$130x10^3$	$80x10^3$
H	Kefir	$35x10^3$	abs	abs	24x10	abs	abs	abs	$73x10^3$	$4x10^3$
	grains	70x10	abs	abs	35x10	abs	abs	abs	$80x10^3$	$4X10^3$

Nutritive growth of the germs on agar

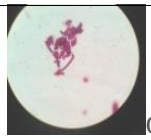
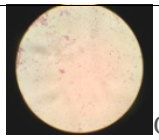
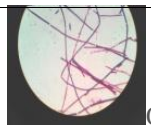
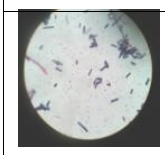
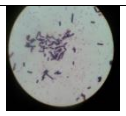
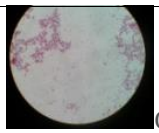
After incubation at room temperature for 24h in a , we found some star shaped-colonies, others were small and fluffy (see Table 7).

Table 7: Microorganisms growth

	P	H
Milk		
Grains		

The various analyses under optical microscope of Gram coloring are represented in Table 8

Table 8: Gram coloration

		Sample P	Sample H
Milk	Star shaped-colonies	 Gram-bacillus and oval yeast	 Gram- cocci
	Fluffy colonies	 Chains of Gram– bacillus and Gram+	 Chains of Gram– bacillus
		 Bacillus Gram + and Gram - individual	 Bacillus Gram + individual and aggregate
Grains	Star shaped-colonies	 Chains of Gram– bacillus	 Gram + cocci individual and aggregate
	Fluffy colonies	 Yeast and Oval Gram - bacillus	 Bacilllus Gram -
		 Bacilllus Gram +	 Bacilllus Gram +in aggregate and Gram –in chains

Auxacolor test: follows in Figure 8:

The reading of micro-plates is made at 48h or 72h if necessary. The aspect of micro-plates at 48h is as

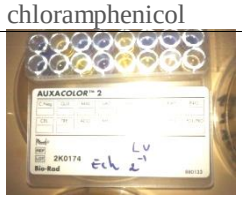
Figure 8: Auxacolor Test at 48h

After 72h, only two micro-plates did not change (sample 2 on medium sabouraud + chloramphenicol and sample 1 on sabouraud), the change in the rest of micro-plates concerns one to two sugars, as follows in Figure 9:

Sample P on sabouraud/ actidion

Sample P milieu sabouraud/ chloramphenicol

Sample H on sabouraud/ actidion



Sample H on sabouraud/ actidion

Figure 10: **Auxacolor Test at 72h**

The codes obtained from the final reading of micro-plates are represented on Table 9 and Figure 11:

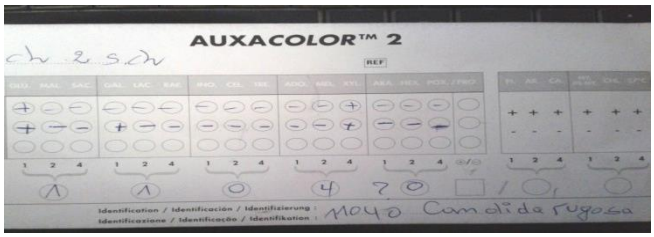


Figure 11: **Auxacolor test codes**

Table 9: Codes and species obtained from Auxacolor Test

	medium	code	Species
Sample P	Sabouraud	11040	Candida rugosa
	Sabouraud/ actidione	57051	Candida kefyr
	Sabouraud / chloramphenicol	13041	Non defined
Sample H	Sabouraud	11051	Candida rugosa
	Sabouraud/ actidione	11050	Candida rugosa
	Sabouraud / chloramphenicol	11040	Candida rugosa

Microscopic results of Rice cream:

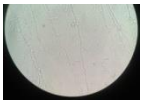
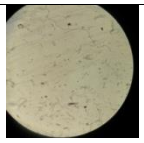
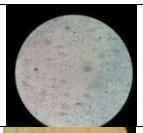
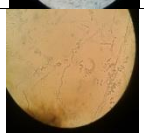
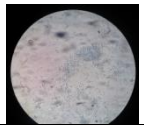
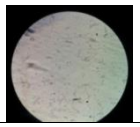
This test did not reveal mycelia forms which justify the presence of the Candida on this medium.

Microscopic results of the direct examination of the colonies in blue cotton:

The reading of the blades under optical microscope with x10 enlargement makes it possible to see various forms of spores, arthrospores (bacterial Spore formed by fragmentation or asexual spore, resulting from the mycelia disintegration of the hyphas), blastospore (a spore resulting from a

budding, synonymous with blastoconidia) and filaments as seen Table 10.

Table 10: mycological study on blue cotton

Sample	Medium	Aspect	Colonies
Sample H	sabouraud + chloramphenicol	Creamy colonies: spore and filaments	
Sample P	sabouraud + chloramphenicol	Creamy colonies :Blastospores and budding yeast	
Sample H	sabouraud	Creamy colonies: Spores	
Sample P	sabouraud	Mould like colonies: arthrospores, tricho-sporic	
Sample H	sabouraud + actidion	spores	
Sample P	sabouraud + actidione	Mould: Filaments and arthrospores	

Discussion

The curve of evolution of pH (Figure.3) enables to note the reduction in pH until stabilization at a constant value where the micro-organisms ferment the totality of lactose and where milk becomes saturated with acids. The threshold of pH under which the pathogenic germs do not develop varies according to the strain and the environment. The behavior of the micro-organisms depends on the food matrix considered. Acetic, lactic, citric and hydrochloric acids for example used to lower the pH do not have the same inhibiting power on the growth of the strains [13].

However, in the absence of data establishing the particular conditions under which the grains of kefir should be maintained, the values threshold of pH diffused by Food and drug administration in the USA were used as bases for the comprehensive assessment of the sensitivity of Kefir grains [14].

Among the most tolerant pathogenic germs to acidity, we note *Staphylococcus aureus, Listeria monocytogenes, Salmonellas sp., Escherichia coli* and *Bacillus cereus* which generally do not develop in pH lower than 4.2; 4.4; 4.5; 4.6 and 4.9 respectively. *Campylobacter jejuni* which is, after *Salmonella sp*., in charge of a large number of declared cases of food infectious disease does not grow normally in a pH lower than 5.5 [15]. Below these limiting values of pH, the medium becomes lethal for these germs and their populations decrease in the time course.

The acidity of kefir samples P and H are 3.4 and 3.27 respectively, indicating the absence of pathogenic germs clostridium; salmonella; shigella; streptococci and staphylococci. Nevertheless, sample P contains some streptococci, which is not necessarily sign of contamination since in literature kefir already contains *streptococci lactis* fermenting milk.

Sample H on chapmann medium was positive with 240 CFU/ml which is lower than the standard of 300 CFU/ml according to the official journal of Democratic Republic of Algeria N° 35. The grains of the studied sample presented a value of 350 CFU/ml which is higher than its fermented milk since it is a set of germs with a higher bacterial load. For sample P we obtained 10 CFU/ml because there were no colonies grown on the surface of the culture medium, only a presence of low yellow color. As we said, staphylococcus do not develop in pH lower than 4.2

and since the samples of 24h of fermentation have a pH lowerthan this value, this confirms a recent contamination from the water used to rinse the grains.

In literature, the microbial composition of kefir grains shows that they contain a diversified microflora with lactic bacteria and yeasts, sometimes associated with acetic bacteria and/or micrococcus. The lactic bacteria include *lactobacilli, pediococcus, lactic hulls, leuconostocs* and *Weissella viridescens*. The microbial groups taking part in the consortium of kefir grains were identified by growth on selective culture media.

The exclusive growth of pediococcus was targeted by the addition of hydrochloride of cysteine, novobiocin and vancomycin in the medium suggested by the International federation of dairy WIRE.

Micrococci are bacteria that contribute to cheese maturation [15]. They have the property of halo-tolerance but require oxygen and less nutritive elements [16]. The choice of the medium was dictated by the dairy application of the basic medium PCA, with skimmed milk.

The selectivity of the medium used to highlight the acetic bacteria is based on their capacity to use alcohol like source of carbon. The lactic bacteria of kefir grains were identified by the analysis of the nucleotidic sequence of ADNr 16S [17].

For lack of means and selective media the molecular based-bacteriological identification was not made, a simple orientation was carried out with Gram coloration which allows to distinguish the shape of bacteria: cocci or bacillus and aggregate or individual and the structure of the membrane.

The Gram negative bacilli found in the samples can be enterobacter or Echerichia coli while the Gram positive maybe lactobacillus. For the cocci, gram negatives are pseudomonadaceae, halobacteraceae, or acidaminococcus fermentans seldom isolated.

Most yeast identified in kefir grains are from candida species, namely *Candida friedrichii, Candida inconspicua, Candida husbands, Candida tenuis, Pichia fermentans, Saccharomyces cerevisiae, Saccharomyces unisporus, Torasporula delbrueckii, Yarrowia lipolytica and Zygosaccharomyces sp* [7].

The modifications in microbial composition of kefir grains are reported in literature to be associated with a rupture in the conditions of culture: freezing of the grains, change of substrate or modification in the composition of milk [21-23].

For maintenance, the rinsing and the draining of the grains before the daily renewal of milk were performed but were not major source of variability for lactobacilli and yeasts. The contact with the air modifies the distribution of microorganisms on the surface. To be functional, the consortium must include bacteria producing kefiran, the typical and constitutive polysaccharide of kefir and of the matrix of the kefir grains.

The strains producing kefiran until now established belong to two taxa which were not identified in the grains in study: *Lactobacillus kefiranofacians* subsp. kefiranofacians and Lb. brevis[23]. We did not find these species; probably the bacteria producing Kefiran in our case are *Lactobacillus brevis* belonging to the Lb species. Kefiri.

Fats of kefir are able to decrease the mutagen effect of pathogens, reducing the oxidative damage induced by a toxic agent. The ingestion of kefir fresh or freeze-dried reduces the immunity response specific to a food allergen. The reduction of blood cholesterol level due to kefir was seen in hamsters with a diet rich in cholesterol. A conducted study in human showed that ingestion during 3 weeks of fermented milk composed of L. *johnsonii* and *bifidobacteries* involves an increase in the total and specific production of IgA in healthy subjects. [24,25].

Conclusion

Kefir is an almost complete nutritive element considered as probiotic useful for the good performance of the organism. This is due to the consortium of bacteria, mould and yeast which restore the body function [26,27] .

Other studies showed a role in the regularization of gastro-intestinal disorders, an anti-tumoral effect thanks to the presence of kefiran which inhibits the growth of tumours and stimulates the humoral immunity in intestinal tissues. Moreover, kefir has a protective effect against the apoptotic destruction of intestinal cells induced by irradiation with X-rays. Kefir could be consumed in the cases of diabetes, obesity or cardiac and renal diseases.

We plan in a future study to focus on the mechanisms by which Kefir and its effective agent; kefiran acts reducing cancer cell growth during tumour development and the biological and physiological changes in the body while ingesting kefir. Kefir may be proposed as natural therapy for diabetes, cancer and other disorders.

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