

## Manufacture of a “Functional” Fermented Milk Product with the Addition of an Alcoholic Plant Origin Extract

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### ABSTRACT

Changes in microbiological, physicochemical and sensory parameters of kefir were studied during refrigerated storage. Kefir batches were prepared with 0 µl/100g (control, K) 15µl/100g (KA) and 30µl/100g (KB) concentration of an ethanolic extract of *Viscum album* and *Abies alba* and samples for analysis were taken 24h after inoculation (day 1), at 10<sup>th</sup> day and 20<sup>th</sup> day of storage at 3 ±1°C. The alcoholic extract resulted from the mixture of leaves and stems from *Viscum album* and *Abies alba* with ethanol in proportion of 1:1:1. The mixture was left at 4°C for one month, filtered and added into the kefir. *Viscum album* is known for its potential immunostimulatory, cytotoxic, proapoptotic and anticancer effects (in-vitro). *Abies alba* is the host of *Viscum album* and their synergy enhance the above properties. The results of this study showed that the use of the ethanolic extract of *Viscum album* and *Abies alba* in kefir production did not alter the microbial and physicochemical characteristics of kefir. It seems that the incorporation of a small concentration (15µl/100g) of the ethanolic extract of *Viscum album* and *Abies alba* in kefir does not affect the final product, which is similar or even better than the control.

**Keywords:** fermented milk products, kefir, *Viscum album*, *Abies alba*.

### INTRODUCTION

Kefir is a fermented dairy product that has its origin in the Caucasian mountains of Russia many centuries ago<sup>1</sup>. It has been widely consumed in Russia and central Asia countries for centuries. Nowadays an increase in kefir consumption in many European countries, Japan and the United States has been reported due to its unique sensory properties and its benefits in health, including antibacterial activity<sup>2</sup>, enhanced immune function<sup>3</sup>, antitumoral activity<sup>4</sup> and hypocholesterolemic effects<sup>5</sup>. This fermented milk product results from the action of different microorganisms present in kefir grains in milk<sup>6</sup>. Kefir grains are whitish or yellowish, irregular granules about the size of a walnut or in some cases, wheat grains. They are insoluble in water and

ordinary solvents. Immersed in milk, kefir grains swell and turn white and initiate the dual lactic acid and alcohol fermentation. Various lactic acid bacteria and yeasts have been identified in kefir grains, including *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus kefir*, *Leuconostoc mesenteroides*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*<sup>7</sup>. The lactic acid bacteria and the yeasts are combined with casein and complex sugars in a polysaccharide matrix. The principal polysaccharide is a water soluble substance known as 'kefiran'. Several homofermentative lactobacillus species including *Lb. kefirifaciens* and *Lb. kefir*<sup>8</sup> produce this polysaccharide. Kefir has a smooth creamy texture, mild acidic taste due to the presence of lactic acid, mild effervescence due to carbon dioxide, and a low concentration of ethanol

produced by yeasts present in the grains. Minor components can also be found, including acetoin, diacetyl, acetaldehyde, and amino acids contributing to the flavour composition<sup>9</sup>.

*Viscum album L.* is a common epiphytic parasite plant, also known as the European mistletoe. The plant is widely distributed in Europe, northwest Africa, southwest and central Asia. It is known for its potential anticancer<sup>10</sup>, antihypertensive<sup>11</sup>, antidiabetic<sup>12</sup>, antioxidant<sup>13</sup>, antimicrobial<sup>14</sup> and antiviral<sup>15</sup> activity. In some countries, especially in Germany, many *Viscum album* preparations, applied mainly in unconventional cancer therapy, are available<sup>16</sup>. *Abies alba* is the host of *Viscum album* and their synergy enhance the above properties<sup>17,18</sup>.

The object of the present study was to assess the effect of the addition of two concentrations of an alcoholic extract of *Viscum album* and *Abies alba* on the physicochemical and sensory characteristics of kefir during storage.

## MATERIALS AND METHODS

### Production of the alcoholic extract

The alcoholic extract resulted from the mixture of leaves and shoots from *Viscum album* and *Abies alba* with ethanol in proportion of 1:1:1. The mixture was left at 4°C for one month, filtered and added into the kefir just after manufacture at two concentrations, a small (15µl/100g) and a high (30µl/100g) one corresponding to kefir A and B respectively.

### Production of kefir

In this study control kefir was manufactured using homogenized cow's milk. The composition of milk was fat 1.68%, lactose 4.87%, total solids 10.49%, protein 3.42%. The milk was boiled at 90°C for 1 min and left to cool at 33-35°C. The DVS freeze-dried mixed mesophilic and thermophilic kefir culture, consisting of *Debaryomyces hansenii*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc* and *Streptococcus thermophilus* (eXact KEFIR 1; Hansen's, Denmark) was added to the milk (10U/100L milk) and the milk was incubated at 33°C until pH decreased to 4.5.

Kefir curd was broken using soft agitation and it was cooled at 4°C by transferring it to cold storage. Kefir was, then, added into glass containers and it was stored at refrigerator temperatures for 20 days.

### Physicochemical and microbial analyses

A pH-meter (Micro pH 2001; Crison, Barcelona, Spain) was used to take pH readings. The titratable acidity of kefir was measured using the Dornic method<sup>19</sup> and the fat content using the Gerber method<sup>20</sup>. Total solids (TS) were determined according to IDF Standard No 4<sup>21</sup> and ash content according to IDF Standard No 27<sup>22</sup>. Total N was measured using the Kjeldahl method<sup>23</sup>. Ethanol content was determined by distillation<sup>24</sup>. Total bacterial counts were measured using Bactoscan FC (Foss Electric, Denmark).

### Colour Measurement

Colour examination of kefir was performed using a Hunter Lab DP-9000 (Hunter Associates Laboratory, Inc., USA) colourimeter. The L\*, a\*, and b\* colour parameters were determined according to the CIELAB colour space, i.e. L\* corresponds to light/dark chromaticity (changing from 0% dark to 100% light), a\* to green/red chromaticity (changing from 60 % green to 60 % red), and b\* to blue/yellow chromaticity (changing from 60 % blue to 60 % yellow). The instrument was calibrated with a black and a white tile before the measurements.

### Sensory evaluation of kefir

Kefir samples were subjected to sensory evaluation after 1, 10 and 20 days of storage at 3°C by a five-member trained panel familiar with dairy products, as described in IDF Standard 99A<sup>25</sup>. Panel members evaluated kefir for appearance and colour, body and texture and flavour using a five-point scale, with 1 being poor, 2 fair, 3 good, 4 very good, and 5 excellent. Panel members were also instructed to report any defects in appearance and colour (e.g. wheying-off, unnatural colour, lack of uniformity, surface discolouration), body and texture (lumpy or granular, slimy, gelatinous, too thin, etc) or flavour (excess acid, yeasty, unclean, etc).

### Statistical analysis

Analysis of variance (ANOVA) using 95% confidence intervals was run on each of the physicochemical and microbiological variables to

disclose possible differences among the samples for the two factors «percentage of added alcoholic extract» and «storage time». All analyses were performed using the software STATGRAPHICS Plus for Windows v.5.2 (1995, Manugistics, Inc, Rockville, MD, USA). The least significant difference of the data is reported ( $P < 0.05$ ).

## RESULT AND DISCUSSION

### Microbial and physicochemical analyses

Figure 1 depicts the changes in the microorganism populations during storage of the kefir. In all sampling days, Total Viable Counts (TVC) did not differ significantly ( $P > 0.05$ ) in the control kefir (K) and in kefir made with the two alcoholic extracts (KA, KB). In the beginning of storage (day 1), the microbial counts ranged between 4.90 and 5.10  $\text{cfu/g} \times 10^7$ . Similar results were reported by other researchers<sup>26,27,28,29,30</sup> but lower levels were recorded by Koroleva<sup>31</sup>. TVC levels decreased significantly ( $P < 0.05$ ) until day 10 of storage and thereafter they levelled off and held steady ( $P > 0.05$ ) until day 20. This pattern of behaviour was observed for all kefir samples i.e., made using the 0  $\mu\text{l}/100\text{g}$  (control, K), 15 $\mu\text{l}/100\text{g}$  (KA) and the 30 $\mu\text{l}/100\text{g}$  (KB) ethanolic extract of *Viscum album* and *Abies alba*. At the end of storage TVC levels ranged between 2.40-2.55  $\text{cfu/g} \times 10^7$  (Figure 1).

Table 1 presents the values of the main physicochemical parameters of the kefir samples made using the two concentrations of the ethanolic extract of *Viscum album* and *Abies alba*, during storage. The pH of kefir decreased and the values of the titratable acidity (TA) increased during storage due to the lactose breakdown by the lactic acid bacteria. The same trend was also observed in other fermented milks like yoghurt<sup>32,33</sup>. The pH and TA values found in this study are considered to be in the acceptable range of a commercial yogurt. According to Chamber<sup>34</sup>, the appropriate range of pH for a commercially available yogurt is between 3.27 and 4.53, and the value of TA is in the range of 0.7% and 1.20%.

Fat, total solids, protein and ash content of control (K), kefir A and kefir B did not differ significantly during storage ( $P > 0.05$ ). Also, the milk from which the kefir was made had almost the

same content ( $P > 0.05$ ) of the above parameters. This finding was consistent with reports by other researchers who observed that the physicochemical composition of fermented milks was the same as that of the source milk<sup>35,36,37</sup>.

Changes in the ethanol concentration of kefir during storage are also shown in Table 1. The alcohol concentration of all samples decreased significantly ( $P < 0.05$ ) during the storage period and kefir B made with the high concentration (30 $\mu\text{l}/100\text{g}$ ) of ethanol extract of *Viscum album* and *Abies alba* showed as expected, the highest concentration of ethanol. General, according to Farnworth<sup>38</sup>, kefir has a low concentration of ethanol because of the action of yeast cells present in the kefir grains.

The results of this study showed that the addition of the alcoholic extract of *Viscum album* and *Abies alba* in the kefir did not influence significantly ( $P > 0.05$ ) the pH and TA values as well as the fat, total solids, protein and ash contents at all sampling days (Table 1).

### Colour measurement of kefir

Colour is an important quality parameter, which along with flavour affect Consumers; preference. Concerning colour parameters (Table 2), the addition of the alcoholic extract of *Viscum album* and *Abies alba* did not significantly ( $P > 0.05$ ) affected the kefir samples except at day 20, in which K kefir showed higher values than the kefir B, for the colour parameter  $L^*$ , looking lighter. Kefir A colour  $L^*$  values ranged in intermediate levels. Therefore, in general, all kefir samples showed the same luminous (parameter  $L^*$ ) and yellow (parameter  $b^*$ ) - green (parameter  $a^*$ ) colour.

The storage time affected, in general, the colour parameter  $a^*$  (Table 2). At day 10, all kefir samples showed lower values than the respected values in days 1 and 20.

### Sensory evaluation of kefir

Table 3 shows the results of the sensory analysis of the kefir samples produced with or without the addition of the ethanolic extract of *Viscum album* and *Abies alba*. All kefir samples showed good acceptability until the end of storage (20<sup>th</sup> day). Katsiari *et al.* (2002)<sup>33</sup>, also, concluded that storage

**Table 1: Changes in physicochemical values in kefir samples made using different amounts of ethanolic extract of *Viscum album* and *Abies alba* during storage**

Age (Days)	Type of kefir	pH	acidity, °D	fat, %	total solids, %	protein %	ash, %	ethanol (v/w)
1	K	4.59±0.03 aA	0.85±0.03 aA	1.83±0.14 aA	10.25±0.3 aA	3.88±0.09 aA	0.72±0.03 aA	0.4±0.0 aA
	KA	4.64±0.01 aA	0.80±0.02 aA	1.76±0.22 aA	9.8±0.77 aA	3.75±0.2 aA	0.76±0.03 aA	0.4±0.0 aA
	KB	4.63±0.10 aA	0.80±0.05 aA	1.85±0.13 aA	9.97±0.57 aA	3.97±0.03 aA	0.75±0.01 aA	0.5±0.1 aA
10	K	4.45±0.01 aAB	0.92±0.02 aAB	2.07±0.03 aA	11.03±0.23 aA	4.08±0.08 aA	0.71±0.02 aA	0.2±0.1 aB
	KA	4.43±0.02 aAB	0.89±0.04 aAB	2.02±0.04 aA	10.92±0.16 aA	4.01±0.09 aA	0.73±0.03 aA	0.2±0.0 abB
	KB	4.42±0.04 aAB	0.9±0.00 aAB	2.08±0.04 aA	11.17±0.15 aA	4.12±0.06 aA	0.73±0.03 aA	0.3±0.0 bB
20	K	4.33±0.08 aB	1.02±0.02 aB	1.85±0.16 aA	9.91±0.50 aA	3.81±0.16 aA	0.73±0.00 aA	0.2±0.0 aB
	KA	4.28±0.04 aB	0.95±0.01 aB	1.82±0.09 aA	9.86±0.61 aA	3.68±0.31 aA	0.73±0.01 aA	0.2±0.0 aB
	KB	4.23±0.02 aB	0.95±0.04 aB	1.86±0.15 aA	9.95±0.43 aA	3.82±0.15 aA	0.73±0.01 aA	0.3±0.0 bB

Data are means of three cheese making trials± standard error.

K: Kefir without an ethanolic extract (control), KA: Kefir with 15µl/100g ethanolic extract of *Viscum album* and *Abies alba*, KB: Kefir with 30µl/100g ethanolic extract of *Viscum album* and *Abies alba*.

a-b: Values in the same row and at the same age, with different letters, differ significantly (LSD test, P<0.05).

A-C: Values in the same row and for the same type of kefir, with different letters, differ significantly (LSD test, P<0.05).

**Table 2: Changes in colour measurements in kefir samples made using different amounts of ethanolic extract of *Viscum album* and *Abies alba* during storage**

Age (Days)	Type of kefir	Colour parameter -L*	Colour parameter -a*	Colour parameter -b*
1	K	94.85±0.81 aA	-4.73±0.07 aA	4.98±0.25 aA
	KA	94.40±0.34 aA	-4.72±0.11 aA	4.97±0.17 aA
	KB	94.47±0.05 aA	-4.80±0.47 aA	4.96±0.07 aA
10	K	97.13±2.32 aA	-7.50±0.28 aB	3.67±0.24 aA
	KA	93.10±0.56 aA	-8.15±0.24 aB	3.71±0.19 aA
	KB	92.09±0.67 aB	-8.45±0.29 aB	3.32±0.49 aA
20	K	94.13±0.15 aA	-5.41±0.99 aAB	4.53±0.40 aA
	KA	93.68±0.08 abA	-5.58±0.74 aA	4.18±0.51 aA
	KB	93.54±0.14 bA	-5.54±0.77 aA	3.59±0.81 aA

Data are means of three cheese making trials± standard error.

K: Kefir without an ethanolic extract (control), KA: Kefir with 15µl/100g ethanolic extract of *Viscum album* and *Abies alba*, KB: Kefir with 30µl/100g ethanolic extract of *Viscum album* and *Abies alba*.

a-b: Values in the same row and at the same age, with different letters, differ significantly (LSD test, P<0.05).

A-C: Values in the same row and for the same type of kefir, with different letters, differ significantly (LSD test, P<0.05)

**Table 3: Organoleptic evaluation of kefir samples made using different amounts of ethanolic extract of *Viscum album* and *Abies alba* during storage**

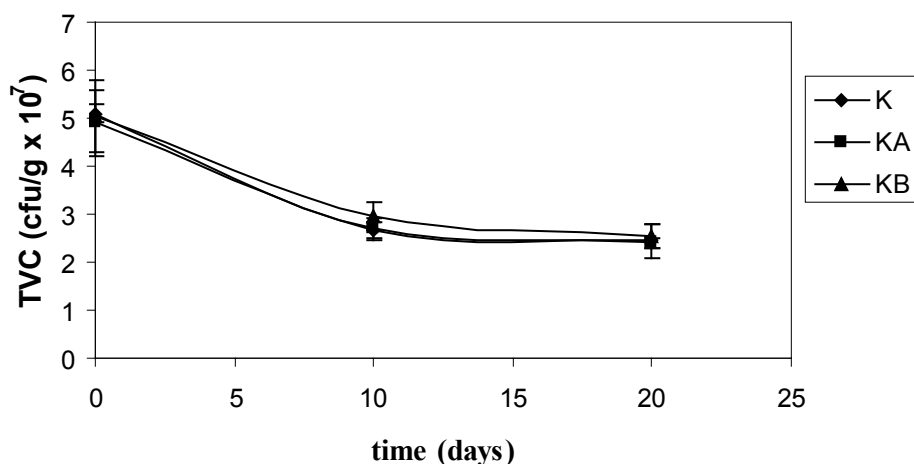
Age (Days)	Type of kefir	Appearance	Texture	Taste
1	K	4.75±0.15 aA	4.6±0.00 aA	4.65±0.05 aA
	KA	4.89±0.09 aA	4.7±0.00 aA	4.68±0.02 aA
	KB	4.80±0.1 aA	4.58±0.12 aA	4.16±0.1 bA
10	K	4.83±0.03 aA	4.75±0.11 aA	4.67±0.09 aA
	KA	4.86±0.00 aA	4.83±0.03 aB	4.77±0.01 aB
	KB	4.83±0.03 aA	4.83±0.03 aA	4.33±0.01 bA
20	K	4.57±0.19 aA	4.53±0.15 aA	4.4±0.16 aA
	KA	4.73±0.05 aA	4.68±0.00 aA	4.59±0.01 aC
	KB	4.73±0.05 aA	4.66±0.08 aA	4.38±0.08 aA

Data are means of three cheese making trials± standard error.

K: Kefir without an ethanolic extract (control), KA: Kefir with 15µl/100g ethanolic extract of *Viscum album* and *Abies alba*, KB: Kefir with 30µl/100g ethanolic extract of *Viscum album* and *Abies alba*.

a-b: Values in the same row and at the same age, with different letters, differ significantly (LSD test, P<0.05).

A-C: Values in the same row and for the same type of kefir, with different letters, differ significantly (LSD test, P<0.05).



**Fig. 1: Changes of Total Viable Counts (TVC) of kefir samples made using different amounts of ethanolic extract of *Viscum album* and *Abies alba* during storage**

did not significantly affect the sensory attributes of yoghurt samples. Opposite results were observed by Kilic *et al.*<sup>27</sup> who found that the scores of all the sensory attributes decreased significantly with time and concluded that kefir kept under refrigeration should be eaten within 3 days of manufacture.

Control kefir (K) and kefir A were very much appreciated by the panellists. The addition of the plant extract did not affect appearance and body and texture of fermented milk product but the addition of the high concentration of *Viscum album* and *Abies alba* ethanolic extract into kefir B resulted in significantly lower flavor scores than the control and Kefir A (Table 3). The panellists noticed rather foreign flavour in kefir B compared to the control and kefir A.

### CONCLUSION

Kefir is a traditional product and its consumption is beneficial to human health. On the

other hand, there appear more and more scientific reports on the possibilities of cancer therapy using mistletoe. The use of a small (15µl/100g) concentration of an ethanolic extract of *Viscum album* and *Abies alba* in kefir production did not alter its physicochemical and sensorial characteristics and kefir containing this extract was very much accepted by the consumers. However, further studies are needed for *in vitro* and *in vivo* experiments to assess its potential use as nutraceutical product.

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