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Evaluation of different preservation techniques on the storage potential of Kefir grains

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Kefir is an acidic, mildly alcoholic dairy beverage produced by the fermentation of milk with a grain-like starter culture (Koroleva, 1988). These grains usually contain a relatively stable and specific balance of microbes that exist in a complex symbiotic relationship (Obermann & Libudzisz, 1998; Witthuhn et al. 2004). The different groups of microbes present in the grains are active at different stages of the fermentation (Koroleva, 1982). The lactococci, including *Lactococcus lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *diacetilactis* provide rapid acid development during the first hours of the fermentation (Litopoulou-Tzanetaki & Tzanetakis, 2000). As the acidity of the milk increases it provides favourable conditions for the growth of the lactobacilli (Rea et al. 1996). The yeasts, acetic acid bacteria and the aroma-producing microbes, mainly leuconostocs, have a much slower growth rate than the lactic acid producers, resulting in the slow production of the aroma compounds and the gradual increase in the concentration of these substances in the later stages of the fermentation (Koroleva, 1982).

In the past the preservation of the microbial populations present in the traditional Kefir grains was achieved by methods including freezing (Garrote et al. 1997), lyophilisation (Oberman & Libudzisz, 1998), air-drying (Kroger, 1993) and refrigeration (Marshall, 1993). Research has shown that traditional Kefir grains preserved by air-drying and lyophilisation retain their activity for up to 12–18 months (Oberman & Libudzisz, 1998). Frozen grains stored at −20 °C were found to maintain the microbial activity for up to 7–8 months, whereas grains stored at refrigerated temperatures showed a decreased activity after about 10 d (Oberman & Libudzisz, 1998).

The aim of this study was to evaluate the impact of four different preservation techniques on the activity of mass cultured Kefir grains (Schoevers & Britz, 2003). The activity of the grains was evaluated at different time intervals using four activity measurements, including changes in substrate pH, titratable acidity (TA), lactose and lactic acid levels of the final Kefir beverage.

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Materials and Methods

Kefir grain activation

Kefir grains were obtained from the Department of Food Science, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa. Fresh, pasteurized, full cream milk purchased at local supermarkets was heat treated at 83–85 °C for 20 min and then cooled to 4 °C. Eighteen grams of grains were aseptically added to 500 ml milk and incubated at 25 °C. After 24 h incubation the grains were recovered from the milk mixture by straining through a sterilised stainless steel kitchen sieve and the grains were then added to pasteurized milk at 25 °C for 24 h. This procedure was repeated seven times prior to the application of the four different preservation techniques.

Preservation techniques

Freshly activated Kefir grains were divided into samples (triplicate units) of 18 g each and were frozen stored (without milk) at −18 °C; refrigerated at 4 °C; air-dried at room temperature for three weeks in a desiccator; and freeze-dried (Heto CT 60e Freeze Dryer, Denmark). After preservation the grains were placed in 100 ml glass containers and stored in moisture tight jars.

Activity tests and mass loss determination

Activity tests were carried out on the Kefir grains directly after the application of the four different preservation techniques (0 months), and after 1, 3 and 10 months storage. All activity tests were done in triplicate. At each time interval, before the activation of the grains, the mass of the preserved grains was determined and the weight loss over the specific storage period calculated.

The activation of the air-dried, lyophilised and refrigerated grains were achieved by the direct transfer of the grains to 500 ml pasteurized, full cream milk at 25 °C, followed by incubation for 24 h. The frozen grains were first allowed to defrost at room temperature before inoculation into milk. All the preserved grains were recovered

Table 1. Mass (g) decrease of 18 g Kefir grains after preservation by the four different preservation techniques over a 10-month storage period

Values are means ± standard deviation for $n=3$

Storage time	Frozen	Refrigerated	Air-dried	Lyophilized
0 Months[1]	18·014 ± 0·014	18·021 ± 0·013	4·216 ± 0·453	3·401 ± 0·184
1 Month	17·969 ± 0·016	17·898 ± 0·214	3·605 ± 0·315	3·490 ± 0·062
3 Months	17·641 ± 0·105	17·306 ± 0·314	2·919 ± 0·112	3·471 ± 0·074
10 Months	17·149 ± 0·441	16·671 ± 1·491	2·639 ± 0·394	3·385 ± 0·201

[1] Directly after preservation

by sieving and then transferred to 1 l pasteurized, full cream milk. The activity tests, including the determination of the pH, TA, lactose utilisation and lactate production in the Kefir beverage were carried out after 0 (fresh milk), 10 and 18 h incubation at 25 °C.

The pH of the Kefir samples was measured using a Knick pH meter (Merck, Cape Town) and the TA (%) was determined by titration of 10 ml Kefir sample (in duplicate for each sample) with standardized 0·1 M-NaOH to the phenolphthalein endpoint (Dixon, 1973). The lactose content was measured colorimetrically with methylamine as indicator (Katsu et al. 1994) and the absorbance of the formed red complex was measured at 540 nm using a Spectronic 20 spectrophotometer (Spectronic Instruments, USA). The lactic acid was measured enzymically with the D-/L+ lactic acid test combination kit (AEC Amersham, Germany).

Statistical analysis

A repeated measures analysis of variance (ANOVA) was done using Statistica™ 6 (version 6.1.409) for Windows™, the sources of variance being the decrease in mass, pH levels, TA, lactose and lactic acid content observed over the 10 month storage period. Mean values were considered significantly different at $P<0·05$. Where interactions were non-significant main effects were interpreted directly and if the samples differed significantly, a Bonferroni multiple comparisons procedure was used to determine which samples differed.

Results and discussion

Morphological characteristics of the preserved grains

The frozen (−18 °C) and refrigerated (4 °C) grains retained the typical appearance, colour and resilient nature of the fresh grains. However, after 10 months storage the refrigerated grains had a clearly detectable acidic and alcoholic odour. This was ascribed to the possible acid and alcoholic fermentation by the microbes present in the moist grains. The lyophilised grains also retained the appearance and colour of the fresh grains, but were found to be extremely brittle. In contrast, the air-dried grains developed a brownish colour and a foul odour, together with a tough structure due to the moisture loss. Mycelial fungal growth on the air-dried grains was observed after approximately two weeks of the drying process.

Mass loss

The mass loss (Table 1) over the 10-month period of the frozen and refrigerated samples was small and not significant, with a total mass loss of 4·8% and 9·2%, respectively. In contrast, the air-drying resulted in a 5-fold (23·4%) decrease in sample mass. However, only a small and not significant decrease in the mass of the air-dried grains was observed over the total storage period once the grains had been dried. In the case of the lyophilised grains (Table 1) an initial mass loss of 81·1% was observed, but the lyophilised grains had a constant mass throughout the rest of the storage period.

Activity tests

pH and TA profiles The pH of fresh milk was 6·53 and served as the control. The pH of the control milk fermented with active Kefir grains decreased to 4·40 during the first 10 h and to 4·13 during the next 8 h of the fermentation.

Similar pH results for the different storage periods (months 0–10) were obtained for the frozen grain samples (Table 2). Freezing has a marked influence on the initial acidification activity of the Kefir grains as was observed directly after preservation (month 0). However, the corresponding pH values of the frozen samples that were stored for periods of up to 10 months were noticeably lower. This is probably due to changes in the starter composition of the preserved (frozen) grains during the storage period, causing the stabilisation of the starter microbes or more active acid-producing bacteria. The storage period had very little effect on the final pH as the pH of all the frozen samples after 18 h fermentation were very similar (~pH 4·30). Freezing may, therefore, be an effective preservation technique of Kefir grains and is in agreement with the results obtained by Garrote et al. (1997).

Refrigeration showed a good retention of the initial grain acidification activity (Table 2). The decrease in the pH over the first 10 h fermentation after 1 month of storage was similar to the decrease in the pH observed directly

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Table 2. The pH and titratable acidity (%) values of the Kefir prepared from the four different preserved grains after 10 h and 18h of fermentation for each storage period

Values are means ± standard deviation for $n=3$

Storage time	Fermentation time (h)	pH				Titratable acidity (%)			
		Frozen	Refrigerated	Air-dried	Lyophilised	Frozen	Refrigerated	Air-dried	Lyophilised
0 Months[1]	10	4·99 ± 0·05	4·57 ± 0·04	4·56 ± 0·06	4·91 ± 0·12	0·73 ± 0·06	0·83 ± 0·05	0·70 ± 0·03	0·81 ± 0·12
	18	4·34 ± 0·03	4·34 ± 0·01	4·24 ± 0·02	4·32 ± 0·04	0·91 ± 0·08	0·93 ± 0·04	0·86 ± 0·01	0·92 ± 0·05
1 Month	10	4·58 ± 0·02	4·51 ± 0·01	4·96 ± 0·16	4·72 ± 0·16	0·77 ± 0·05	0·78 ± 0·01	0·58 ± 0·05	0·72 ± 0·03
	18	4·29 ± 0·02	4·30 ± 0·01	4·44 ± 0·03	4·32 ± 0·05	0·94 ± 0·01	0·90 ± 0·01	0·87 ± 0·08	0·92 ± 0·04
3 Months	10	4·59 ± 0·06	5·21 ± 0·16	4·42 ± 0·07	5·77 ± 0·07	0·81 ± 0·02	0·77 ± 0·02	0·71 ± 0·02	0·39 ± 0·02
	18	4·29 ± 0·01	4·39 ± 0·11	4·22 ± 0·03	4·67 ± 0·01	0·87 ± 0·04	0·79 ± 0·06	0·82 ± 0·04	0·73 ± 0·08
10 Months	10	4·43 ± 0·01	4·32 ± 0·04	5·36 ± 0·19	5·48 ± 0·19	0·69 ± 0·01	0·72 ± 0·06	0·25 ± 0·07	0·26 ± 0·08
	18	4·23 ± 0·03	4·15 ± 0·02	4·24 ± 0·18	4·37 ± 0·19	0·45 ± 0·01	0·51 ± 0·10	0·39 ± 0·02	0·41 ± 0·06

[1] Directly after preservation

Table 3. The lactose and lactic acid content (g.100 g^{-1}) of Kefir prepared with the four different preserved grains stored for 10 months after 10 h and 18 h fermentation

Values are means ± standard deviation for $n=3$

Compound (g.100 g^{-1})	Fermentation time (h)	Frozen	Refrigerated	Air-dried	Lyophilised
Lactose	10	3·85 ± 0·37	3·90 ± 0·09	3·76 ± 0·47	3·57 ± 0·32
	18	3·62 ± 0·47	3·77 ± 0·29	3·47 ± 0·29	3·35 ± 0·18
Lactic acid	10	0·53 ± 0·01	0·51 ± 0·03	0·26 ± 0·02	0·34 ± 0·05
	18	0·60 ± 0·06	0·55 ± 0·04	0·60 ± 0·01	0·61 ± 0·03

after preservation. However, after 3 and 10 months storage the decrease in the pH after 10 h fermentation differed markedly. These varying pH values for the refrigerated grains are possibly due to changes in the microbial activity of the preserved grains over the 10-month storage period. The pH of the samples after 18 h fermentation was similar for all the stored samples. From the data it is clear that refrigerated grains retained the ability to reach a low final fermentation pH throughout the 10-month storage period, in accordance with results of Pintado et al. (1996).

The air-dried grains showed a good retention of the initial acidification activity (Table 2). After 1 month storage, a decrease in the activity of the grains was observed. After 10 months the microbial activity of the grains decreased as only a small decrease in pH over the first 10 h fermentation was observed. However, the microbial activity appeared to recover over the 18 h fermentation time, suggesting that a change in the population had occurred, but the acid producers were able to recover over the longer incubation time.

The lyophilised grains showed the lowest retention of the activity (Table 2). After 3 and 10 months storage a decrease in the activity of the lyophilised grains was observed and the decrease in the pH over the first 10 h fermentation was significantly slower than for the frozen and refrigerated grains. This slow initial rate of acid production by the dried (lyophilised and air-dried) grains could be ascribed to a prolonged metabolic lag-phase induced by the dehydration of the grains (Tamime, 1981). This prolonged lag time was expected as microbes in a dried state take a considerable time to re-adapt their metabolism to biomass production (Pintado et al. 1996). The total decrease in the pH after 18 h fermentation is in the same order for all the preserved samples, indicating a rapid acid production by the remaining lactic acid bacteria, particularly in the dehydrated samples. The retention of the acidification activity in the dried grains (air-dried and lyophilised) is consistent with the findings by Oberman & Libudzisz (1998) who reported that dried grains remain active for a period of 12–18 months.

The TA of fresh milk was found to be 0·22% and after 10 h fermentation with active Kefir grains it reached 0·76% and then increased to 0·88% after 18 h. The TA profiles of the milk after 10 h and after 18 h fermentation with the frozen and the refrigerated grains were similar directly after preservation and after 1 and 3 months storage (~0·77%), but were lower after 10 months of storage. The TA values for the air-dried and the lyophilised grains directly after preservation were comparable with the values of the active grains. However, after 10 months storage a significant decrease ($P=0·016$ for the air-dried and $P=0·001$ for the lyophilised grains) in TA values was observed (Table 2). These low TA values were probably due to a prolonged lag phase of the Kefir grain microbes and the consequent decreased activity of the starter microbes present in the dried grains. In addition, the low TA values

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can also possibly be ascribed to a lower number of viable bacterial cells present in the dried grains at the end of the 10-month storage period. This loss in acidification activity by the Kefir grains could also be due to damage and even cell death caused by the extreme dehydration and the extensive storage period of the grains.

Lactose utilisation and lactate production. The lactose content of the Kefir samples prepared using the Kefir grains that were stored for 10 months are shown in Table 3. The unfermented milk contained, on average, 5·00 g lactose 100 g^{-1} Kefir. The data clearly indicates that, with lactose utilisation taken as the measure of activity, no significant difference between the final values of the different preservation techniques after 10 months storage was observed.

The highest production of lactic acid was observed for the frozen (P=0·002) and the refrigerated (P=0·006) grain samples (Table 3) after 10 h fermentation. The air-dried and lyophilised grains showed a significantly slower initial lactic acid production. However, after 18 h fermentation the lactic acid content of the Kefir samples were similar, indicating a relative similar activity for all of the preserved grain samples at the end of the fermentation period. The highest lactic acid production for this time period was recorded for the lyophilised grains, followed by the frozen grains, air-dried grains and refrigerated grains.

The use of Kefir grains as a starter culture for the production of Kefir has widespread possibilities in Southern Africa, but a reliable and economic grain preservation technique is required to facilitate the successful marketing of this unique fermented beverage. Based on the retention of the acidification activity in the preserved Kefir grains, all four of the preservation techniques proved to be suitable for the preservation of the grains. However, air-dried grains are not recommended for commercialisation due to the unacceptable colour and smell of the grains. Freezing and refrigerated storage showed a good retention of the acidification activity throughout the 10-month storage period. The storage period had no significant influence on the activity of the frozen and refrigerated grains and both these preservation techniques resulted in a low final pH (∼4·3) throughout the 10-month storage period. The air-drying and lyophilisation techniques also proved to be successful in retaining the acidification activity of the grains, however, an increased lag phase and a lower initial rate of pH decrease were observed for both techniques with the prolonged storage period.

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