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Note

Effect of storage temperature on beneficial microbial load in rainbow trout feed supplemented with *kefir*

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ABSTRACT

Effect of different storage temperatures on beneficial microflora in rainbow trout feed supplemented with varying levels of *kefir*, produced using natural *kefir* grain was investigated. Three different feed samples were prepared using 2, 5 and 10% *kefir* supplementation into basal practical feed. Basal feed without *kefir* served as control. The feed samples prepared were stored in air-tight plastic bags at 24ºC, 4ºC and -20ºC for 28 days. *Lactobacillus* spp., *Lactococcus* spp., *Lactobacillus acidophilus*, *Bifidobacterium* spp. and yeast content of feeds stored at different temperatures were analysed on 1st, 7th, 14th, 21st, and 28th day of storage. Microbial counts in feed samples stored at 24ºC up to 28 days were found lower than those stored at 4ºC and -20ºC. Feed samples stored at 4ºC and -20ºC showed similar results pertaining to levels of microorganisms. Results showed that 5% *kefir* supplemented feed had the highest level of beneficial microbes which were able to survive at 4°C during 28 days of storage.

Keywords: Feed, *kefir*, Probiotics, Rainbow trout, Storage period, Storage temperature

Probiotics are live microorganisms supplemented in food or feed which provide beneficial effects on the intestinal microbial balance. The use of microbial probiotics such as *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Carnobacterium, Shewanella, Bacillus, Aeromonas, Plesiomonas, Vibrio, Enterobacter, Pseudomonas, Clostridium, Saccharomyces, Fusobacterium* and *Eubacterium* in aquaculture is widely accepted (Gatesoupe, 1999; Irianto and Austin, 2002a, b; Balcazar *et al.,* 2006). *Kefir* is a fermented diary beverage made with *kefir* grains (a yeast/bacterial fermentation starter) that is rich in natural probiotics such as *Bifidobacterium* spp. and *Lactobacillus acidophilus*. It is an acidic, viscous, slightly carbonated fermented milk drink having a variety of health benefits (Ozer and Kırmacı, 2010; Guzel-Seydim *et al.,* 2011). Therapeutic properties of *kefir* on immune and digestive systems have been well documented (Guzel-Seydim *et al.,* 2011); however, there is no study related to *kefir* as a supplement in fish feed. Various parameters such as storage conditions and period of storage affect total microbial content of the feed when supplemented with probiotics. The objective of the present study was to evaluate the effects of different storage period and temperatures on microbial content of rainbow trout feed supplemented with *kefir* produced using natural *kefir* grains.

Kefir grains were obtained from Suleyman Demirel University, Department of Food Engineering, Isparta in Turkey. In the laboratory, *kefir* grains were inoculated (2%, w/v) into pasteurised milk at 24°C for 22 h to produce *kefir*. At the end of fermentation (pH 4.6) the grains were retrieved by sieving and *kefir* was stored at 4°C for 1 day. Commercial rainbow trout feed (crude protein 45%, crude lipid 20%, and digestible energy 4325 kcal kg^{-1}) was used in this study. *Kefir* was supplemeted at levels of 2, 5 and 10% to the basal practical feed. Feed without *kefir* fortification served as control feed. The feed was ground and sieved through a 320 µm mesh to get fine powder. The resulting feed samples were homogenised with 40% water-*kefir* mixture of the total feed weight. The prepared feed samples were pressure pelleted using a meat grinder (2 mm die) and dried in cool air for 24 h. The pelleted feeds were then crumbled using a mortar and pestle, sieved through a 2 mm mesh and stored in air tight plastic bags. Feed samples were stored at 24ºC, 4ºC and -20ºC for 28 days in triplicates. Total mesophilic bacteria, *Lactobacillus* spp., *Lactococcus* spp., *Lactobacillus acidophilus*, *Bifidobacterium* spp. and yeast were enumerated on 1st, 7th, 14th, 21st and 28th days of storage from each sample (triplicates).

Viable bacteria and yeast counts of feed samples supplemented with *kefir* were determined by plating appropriate dilutions on agar plates. Differential enumeration was performed on Plate count agar (PCA) for total mesophilic bacteria, MRS (de Man, Rogosa and Sharpe Agar) agar for *Lactobacillus* spp., MRS-salicin agar for *L. acidophilus*, M17 agar for *Lactococcus* spp., MRS-NNLP agar (neomycinsulfate, 100 mg l^{-1}, nalidixicacid, 50 mg l^{-1}, lithium chloride 3000 mg l^{-1}, paromomycin sulfate 200 mg l^{-1}) for *Bifidobacterium* spp. and PDA (potato dextrose agar) for yeasts. The incubation conditions for each microorganism are summarised in Table 1. By taking into account the dilution factor, the number of viable microorganisms was expressed as colony forming units (cfu) per g (Collins and Lyne, 1976; Austin and Austin, 1989). The comparisons of data between groups were made using one way analysis of variance (ANOVA). The analyses were performed using SPSS 15.0 (SPSS INC. Chicago, IL, USA) program.

The stability of probiotics is influenced by various environmental factors including the species, strain, biotype, water activity, temperature, pH, osmotic pressure, mechanical friction and oxygen (Wang *et al.*, 2008). In our study, microorganisms of feed samples stored at different temperatures (24, 4 and -20°C) were determined on 1st, 7th, 14th, 21st and 28th days of storage (Tables 2, 3, 4). Our results showed that only feed samples supplemented with kefir were positive for the beneficial microbial flora *viz., Lactobacillus* spp., *Lactococcus* spp., *L. acidophilus*, *Bifidobacterium* spp. and yeast. Feed samples supplemented with *kefir* (5%) contained 7.75 log cfu g^{-1} *Lactobacillus* spp., 7.71 log cfu g^{-1} *Lactococcus* spp., 7.65 log cfu g^{-1} *L. acidophilus*, 4.50 log cfu g^{-1} *Bifidobacterium* spp. and 4.52 log cfu g^{-1} yeast at 24°C at day 1 (Table 3). The control sample which had no *kefir,* did not carry any of these useful bacteria and yeast. *Kefir* inoculated feed samples kept at 4°C and -20°C had higher microbial counts after the storage period of 28 days. However the load of *Lactobacillus* spp., *Lactococcus* spp., *L. acidophilus*, *Bifidobacterium* spp. and yeast in kefir inoculated feed samples that were kept at 24°C, for all *kefir* supplementation levels, significantly declined at the end of the 28 days of storage ($p<0.05$). Supplementation of feed with 5% natural kefir led to sufficient levels of beneficial microbes and the microbes were able to survive at 4°C for 28 days of storage.

An initial decline was seen in the numbers of *L. acidophilus* of 1, 0.49 and 0.42 log cfu g^{-1} in feed samples supplemented with 2% *kefir* at 24, 4 and -20°C, respectively during 28 days storage. Our finding agrees with that of Robertson *et al.* (2000). Furthermore,

Table 1. Incubation conditions of microorganisms

Microorganisms	Incubation temperature (°C)	Incubation time (days)	Anaerobic incubation (6% CO_2)
Lactococcus spp.	37	3	+
Lactobacillus spp.	37	3	+
L. acidophilus	37	3	+
Bifidobacterium spp.	37	3	+
Total aerobic mesophilic bacteria	35	2	-
Yeast	25	5	-

Table 2. Microbial counts of feed samples supplemented with 2% *kefir* at different storage temperatures (log cfu g^{-1})

Duration (days)	1				7				14				21				28			
Storage temp.(°C)	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM
Lactococcus spp.	6.24^{aA}	6.54^{aA}	6.48^{aA}	0.16	5.63^{bAB}	6.36^{aA}	6.36^{aA}	0.15	4.99^{bB}	6.12^{aA}	6.16^{aA}	0.24	5.01^{bB}	6.37^{aA}	6.33^{aA}	0.26	5.43^{aAB}	6.13^{aA}	6.61^{aA}	0.25
Lactobacillus spp.	6.39^{bA}	6.65^{abA}	6.92^{aA}	0.10	5.81^{bB}	6.46^{aA}	6.18^{abBC}	0.12	4.08^{cD}	6.02^{aB}	5.30^{bD}	0.36	4.95^{bC}	6.07^{aB}	6.03^{aC}	0.23	5.07^{cC}	6.08^{bB}	6.37^{aB}	0.25
L. acidophilus	6.15^{bA}	6.49^{abA}	6.80^{aA}	0.12	5.63^{bAB}	6.28^{aAB}	6.64^{aA}	0.19	5.00^{cC}	6.37^{aAB}	5.77^{bA}	0.25	5.19^{bBC}	6.25^{aB}	6.27^{aA}	0.22	5.15^{bBC}	6.00^{aC}	6.38^{aA}	0.23
Bifidobacterium spp.	4.70^{aA}	4.62^{aA}	4.88^{aA}	0.07	3.61^{cB}	4.05^{bA}	5.09^{aA}	0.27	2.74^{cC}	4.54^{aA}	3.92^{bC}	0.33	2.15^{bD}	3.67^{aB}	4.04^{aBC}	0.36	ND^{cE}	2.65^{bC}	4.24^{aB}	0.78
Yeast	4.09^{aA}	3.66^{aAB}	3.77^{aB}	0.09	3.83^{bB}	3.80^{bA}	4.02^{aA}	0.04	3.34^{aC}	3.74^{aA}	3.61^{aB}	0.08	3.33^{bC}	3.84^{aA}	3.39^{bC}	0.10	3.56^{aC}	3.23^{aB}	3.71^{aB}	0.10
Total microorganisms	6.08^{aA}	6.57^{aA}	6.50^{aAB}	0.13	5.72^{bA}	6.26^{abA}	6.54^{aA}	0.16	4.99^{bB}	6.36^{aA}	6.18^{aBC}	0.27	4.94^{cB}	6.42^{aA}	6.14^{bC}	0.28	5.13^{cB}	6.09^{bA}	6.71^{aA}	0.29

$^{a-e}$Values in the same row with different superscripts on the same day at different temperatures are significantly different from each other ($p<0.05$).
$^{A-E}$Values in the same row with different superscripts at constant temperature on different days are significantly different from each other ($p<0.05$).
SEM: Standard Error of Means; ND: Not Determined.

Table 3. Microbial counts of feed samples supplemented with 5% *kefir* at different storage temperatures (log cfu g^{-1})

Duration (days)	1				7				14				21				28			
Storage temp. (°C)	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM
Lactococcus spp.	7.71^{aA}	7.05^{abA}	6.74^{bB}	0.19	5.30^{bB}	6.57^{aAB}	6.71^{aB}	0.29	4.63^{bC}	6.52^{aB}	6.60^{aB}	0.40	4.54^{cC}	6.53^{bB}	6.58^{aB}	0.42	5.80^{cB}	6.71^{bAB}	7.18^{aA}	0.25
Lactobacillus spp.	7.75^{aA}	6.76^{bA}	6.75^{bB}	0.22	5.86^{cB}	6.04^{bC}	6.52^{aB}	0.12	4.29^{cE}	6.22^{aBC}	5.72^{bD}	0.36	4.54^{cD}	6.46^{aABC}	6.10^{bC}	0.37	5.73^{cC}	6.62^{bAB}	7.25^{aA}	0.27
L. acidophilus	7.65^{aA}	6.65^{bA}	6.89^{bB}	0.19	5.75^{bB}	6.75^{aA}	6.89^{aB}	0.23	4.78^{bC}	6.12^{aA}	6.27^{aD}	0.32	4.78^{bC}	6.51^{aA}	6.45^{aC}	0.35	5.88^{cB}	6.62^{bA}	7.37^{aA}	0.27
Bifidobacterium spp.	4.50^{bA}	4.79^{aAB}	4.55^{abB}	0.06	3.71^{bB}	4.63^{aB}	5.14^{aA}	0.27	3.04^{cC}	4.94^{aA}	4.48^{bBC}	0.36	2.39^{bD}	3.86^{aC}	4.09^{aCD}	0.33	ND^{bE}	3.84^{aC}	3.74^{aD}	0.79
Yeast	4.52^{aA}	4.23^{abA}	3.95^{bB}	0.11	4.12^{cB}	4.26^{bA}	4.40^{aA}	0.05	3.85^{bC}	4.12^{aA}	3.94^{abB}	0.05	3.88^{aC}	3.98^{aA}	4.11^{aB}	0.04	3.73^{bD}	4.02^{bA}	4.41^{aA}	0.13
Total microorganisms	7.85^{aA}	7.06^{bA}	6.84^{cCD}	0.19	5.84^{bB}	6.81^{aAB}	6.98^{aB}	0.22	4.87^{bC}	6.64^{aAB}	6.72^{aD}	0.38	4.88^{bC}	6.83^{aB}	6.92^{aB}	0.42	6.01^{bB}	6.21^{abB}	7.44^{aA}	0.30

$^{a-e}$Values in the same row with different superscripts on the same day at different temperatures are significantly different from each other ($p<0.05$).
$^{A-E}$Values in the same row with different superscripts at constant temperature on different days are significantly different from each other ($p<0.05$).
SEM: Standard Error of Means; ND: Not Determined.

Table 4. Microbial counts of feed samples supplemented with 10% *kefir* at different storage temperatures (log cfu g^{-1})

Duration (days)	1				7				14				21				28			
Storage temp. (°C)	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM	+24	+4	-20	SEM
Lactococcus spp.	6.91^{bA}	7.94^{aA}	7.95^{aA}	0.22	5.57^{bB}	7.67^{aB}	7.10^{aB}	0.40	5.35^{bB}	6.69^{aC}	6.76^{aD}	0.29	5.57^{bB}	6.59^{aC}	6.61^{aE}	0.21	5.61^{bB}	6.67^{aC}	6.90^{aC}	0.25
Lactobacillus spp.	6.93^{bA}	7.97^{aA}	7.86^{aA}	0.20	5.92^{cB}	7.52^{aB}	6.77^{bB}	0.29	5.08^{cD}	6.35^{aD}	5.90^{bD}	0.23	5.52^{cC}	6.60^{bC}	6.23^{aC}	0.20	5.60^{bC}	6.69^{aC}	6.46^{aBC}	0.21
L. acidophilus	6.91^{bA}	7.90^{aA}	7.78^{aA}	0.20	5.81^{cB}	7.68^{aB}	7.22^{bB}	0.35	5.64^{bB}	6.69^{aC}	6.34^{aD}	0.19	5.72^{bB}	6.48^{aD}	6.32^{aD}	0.14	5.71^{bB}	6.71^{aC}	6.64^{aC}	0.20
Bifidobacterium spp.	4.39^{bA}	4.50^{bB}	4.67^{aB}	0.05	3.74^{cB}	4.79^{bA}	5.19^{aA}	0.27	3.28^{bC}	4.56^{aB}	4.34^{aBC}	0.26	3.56^{bBC}	3.88^{aC}	4.02^{aC}	0.08	ND^{bD}	3.92^{aC}	3.87^{aC}	0.82
Yeast	4.59^{aA}	4.66^{aB}	4.58^{aA}	0.06	4.44^{bA}	4.85^{aA}	4.63^{abA}	0.07	3.93^{bC}	4.50^{aC}	4.06^{bA}	0.11	4.24^{bB}	4.55^{aBC}	4.28^{bAB}	0.06	3.95^{bC}	4.16^{aD}	4.12^{aA}	0.04
Total microorganisms	6.75^{bA}	7.95^{aB}	8.03^{aA}	0.26	6.06^{cB}	7.76^{aA}	734^{bB}	0.32	5.68^{bC}	6.76^{aB}	6.74^{aE}	0.22	5.65^{bC}	6.75^{aB}	7.05^{aC}	0.27	5.74^{bBC}	6.71^{aB}	6.88^{aD}	0.22

$^{a-e}$Values in the same row with different superscripts on the same day at different temperatures are significantly different from each other ($p<0.05$).
$^{A-E}$Values in the same row with different superscripts at constant temperature at different days are significantly different from each other ($p<0.05$).
SEM: Standard Error of Means; ND: Not Determined.

decrease in the counts of *Bifidobacterium* spp. in feed samples supplemented with 5% *kefir* after 28 day storage. It was found that 5% *kefir* inoculated feed sample kept at 24°C had no *Bifidobacterium* spp. at day 28 whereas a 4°C and -20°C, significantly higher counts of *Bifidobacterium* spp. ($p<0.05$) was observed. Similar results were observed for 2% (Table 2) and 10% *kefir* (Table 4) inoculated feed samples. There were no differences in *Lactobacillus* spp. counts in feed samples supplemented with 10% *kefir* stored at different temperatures. Other studies also have reported decline in probiotic content of feeds depending on the storage temperature and period (Robertson *et al.,* 2000; Irianto and Austin, 2002a).

According to our findings, it was concluded that the feed supplemented with 5% *kefir* had significantlty high content of beneficial microorganisms which were able to survive at 4°C for 28 days of storage. This needs to be evaluated at a larger scale before it can be suggested for use in fish feed.

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