

EMIKO Vital – A Fermentation Drink with Beneficial Effects on Intestinal Health

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ABSTRACT

Background: The intact intestinal barrier has several important immunological and non-immunological functions and plays a key role in intestinal and systemic health. The epithelial cell layer is one of the most important non-immunological components as it provides a physical barrier between the contents of the intestinal lumen and the rest of the body. It ensures efficient absorption of essential nutrients from the intestinal lumen and produces mucus and substances with regulatory properties.

Experimental: In the present study we investigated the antioxidant, regenerative and anti-inflammatory potential of a new fermentation drink named EMIKO Vital on cultured porcine intestinal epithelial cells (cell line IPEC-J2) and functional neutrophils (differentiated human promyelocytes; cell line HL-60). The active ingredients of fermentation drinks are known for offering significant health benefits and can improve gastrointestinal health, strengthen the immune system and reduce the risk of chronic diseases.

Results: The antioxidant effect of EMIKO Vital increased in a dose-dependent manner and reached its maximum at the highest test concentration of 20 mg/ml with an inactivation of free radicals of over 80%. The antioxidant effect was already statistically significant at the lowest EMIKO Vital concentration of 1 mg/ml ($p \leq 0.01$; Wilcoxon-Mann-Whitney test). Exogenous oxidative stress without protection by EMIKO Vital resulted in a cell viability of only $43.7 \pm 4.6\%$ (mean value $\pm$ standard deviation), while the cell viability with protection by EMIKO Vital was $85.3 \pm 8.9\%$ (mean value $\pm$ standard deviation; $p \leq 0.01$; Wilcoxon-Mann-Whitney test). Moreover, EMIKO Vital significantly improved the regeneration of intestinal epithelial cells in a dose-dependent up to a test concentration of 10 mg/ml. For EMIKO Vital concentrations of 5 mg/ml and 10 mg/ml, the difference in the residual cell-free space to the controls was statistically significant ($p \leq 0.01$, Wilcoxon-Mann-Whitney test). The addition of EMIKO Vital caused a dose-dependent inhibition of endogenous radical generation by inflammation-mediating cells. From a concentration of 5 mg/ml, this inhibition was statistically significant ($p \leq 0.01$) and ranged from 25% at 5 mg/ml to almost 50% at 20 mg/ml.

Conclusions: In summary, the fermentation drink EMIKO Vital significantly compensated local oxidative stress and the associated consequences such as cell damage, delayed regeneration/healing, inflammatory processes and non-specific irritation of the intestine. It also promoted the cell regeneration/wound healing process. The results of the present study give a first insight on the potential of the fermentation drink to promote and maintain intestinal health and thus systemic health *in vivo*.

Keywords

Intestinal epithelial cells

IPEC-J2

Regeneration

Oxidative stress

Antioxidant

Reactive oxygen species

HL-60

Neutrophil

Inflammation

Research Article

INTRODUCTION

The intact intestinal barrier has several important immunological and non-immunological functions. The epithelial cell layer is one of the most important non-immunological components as it provides a physical barrier between the contents of the intestinal lumen and the rest of the body. It ensures efficient absorption of essential nutrients from the intestinal lumen and produces mucus and substances with regulatory properties. Intestinal hyperpermeability, due to reduced integrity of the junctional complexes between neighboring epithelial cells [1], has been shown to contribute to the pathogenesis of several gastrointestinal diseases, thus affecting not only intestinal health but also systemic health [2-4]. Ingested substances and microbial pathogens that disrupt normal cellular homeostasis in the intestine can cause oxidative stress and gastrointestinal damage due to an excess of reactive oxygen species [5,6].

Fermentation, a traditional method originally used for food preservation and storage, has evolved over time with advances in technology and a deeper understanding of the process. Today, fermentation is mainly used to improve food quality, enhance flavour and increase nutritional value [7,8]. The active ingredients of fermentation drinks are known for offering significant health benefits and can improve digestive health, strengthen the immune system and reduce the risk of chronic diseases [9,10].

Prompted by this background we investigated the antioxidant, regenerative and anti-inflammatory potential of a new fermentation drink named EMIKO Vital on cultured intestinal epithelial cells and functional neutrophils. The results of the present study might give a first insight on the potential of the fermentation drink to promote and maintain intestinal health and thus systemic health *in vivo*.

MATERIAL AND METHODS

EMIKO Vital

EMIKO Vital is a fermentation drink which - according to the manufacturer - is vegan, is gluten-free, lactose-free and histamine-free and contains lactic acid bacteria, yeasts, bifidobacteria, Akkermantia muciniphila (metabolic products), acacia fiber, grape seed flour, yacon syrup, Reishi-Ling-Zhi-Ganoderma lucidum and Hericium erinaceus as bioactive ingredients. The drink was provided by the manufacturer, EMIKO Gesellschaft für Umwelttechnologie mbH, D - 53340 Meckenheim, Germany, for the *in vitro*-study presented here.

The manufacturer's recommended intake is 2 x 25 ml EMIKO Vital per day. This corresponds to a concentration of

20 mg/ml in the intestine, 10 mg/ml in the blood and 2 mg/ml in the body fluid (the latter two with a theoretically calculated bioavailability of 100%). According to these calculated concentrations, EMIKO Vital was tested in cell cultures in the concentration range between 0 (= control) and 20 mg/ml. The fermentation drink was prepared in phosphate buffered saline as a 10-fold concentrated suspension and shaken several times before added to the cell cultures or the reaction mixture in all subsequent pipetting steps.

Cell culture

The investigations were conducted with two cell types:

1. Porcine intestinal epithelial cells (cell line IPEC-J2; ACC-701; Leibniz Institut, DSMZ, Braunschweig, Germany). The cells were routinely grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% growth mixture and 1% antibiotics and cultivated in an incubator at 37°C with an atmosphere of 5% CO₂ and 95% air at nearly 100% humidity. The cells were routinely cultivated as mass cultures and were regularly subcultured twice a week with fresh culture medium. For the experiments, cells were taken from 80-90% confluent mass cultures.
2. Human promyelocytes (cell line HL-60; ACC-3; ECACC 98070106; Leibniz Institut, DSMZ, Braunschweig, Germany). The non-adherent cells were routinely cultured as suspension cultures in RPMI 1640 medium supplemented with 10% growth mixture and 1% antibiotics in an incubator at 37°C with an atmosphere of 5% CO₂ and 95% air at nearly 100% humidity and were subcultured twice a week. At specific culture conditions with 1.5% dimethyl sulfoxide in the culture medium, the cells were differentiated into so-called functional neutrophils within six days. Neutrophils are able to migrate from the blood into a previously damaged and inflamed area of tissue, where they generate a local excess of superoxide anion radicals in the course of an oxidative or respiratory burst, thus delaying successful tissue healing [11-13].

Examination of antioxidant potential

Independent of the investigations with organ-specific cell cultures, the antioxidant (= radical scavenging) effect of the fermentation drink was investigated. For this purpose, EMIKO Vital was added in various concentrations to a reaction mixture consisting of water and a water-soluble tetrazolium dye (WST-1; Sigma-Aldrich, Deisenhofen, Germany) as well as superoxide anion radicals from a stock solution of potassium

peroxide. The dye is cleaved by the radicals present in the reaction mixture and changes its color. This color change can be measured and used for the evaluation of the inactivation/neutralization of the radicals in the reaction mixture. The color change was measured with an Elisareader (BioTek ELx 808 with software Gen 5 version 3.00) as a differential measurement at $\Delta OD = 450 - 690$ nm at definite time points up to 30 min. The analysis was carried out using Microsoft Excel. A total of four independent experimental series was conducted with two replicates per test concentration ($n=4$).

Examination of cell viability after oxidative stress

Intestinal epithelial cells were seeded at a density of 100,000 cells/well in 96-well plates and were allowed to attach, spread and stabilize their metabolism for 48 hours. Then, cells were incubated with various concentrations of hydrogen peroxide (0 to 2 mM) and various concentrations of EMIKO Vital (0 to 20 mg/ml) for another 24 hours. The surviving cells were measured by a redox color reaction after the addition of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; Xenometric, Allschwil, Switzerland) with an Elisareader (BioTek ELx 808 with software Gen 5 version 3.00) as a differential measurement at $\Delta OD = 450-690$ nm at definite time points up to 120 min. XTT is cleaved by metabolically active cells and, therefore, represents cell viability. Three independent experiments were conducted ($n=3$).

Examination of cell regeneration/wound healing

The granulation phase, characterized by the occurrence of migration and proliferation of epithelial cells for closing a defect after injury [14,15], was simulated. For this purpose, cells were seeded at a density of 200,000 cells/ml into the individual compartments of a silicone 4 well-culture insert (ibidi, Gräfelting, Germany). The single compartments of the inserts are separated by a 500 μ m thick silicone bar with an outer silicone frame of 700 μ m. Due to the special adhesion area, a silicone insert adheres firmly to the bottom of a culture dish and forms a distinct cell-free area (artificial wound), which the cells can colonize by migration and proliferation.

Upon reaching confluency within 48 hours after intestinal epithelial cell seeding, the silicone frames were removed with tweezers to achieve a sharp edge of the cell-free area between the compartments. 0 (= control) to 20 mg/ml EMIKO Vital were added to the cells in separated dishes. Cells were allowed to migrate and proliferate for 10 hours. Finally, cell cultures were fixed with 100% methanol, stained with Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany) and air-dried. The colonized area was examined by micrographs and calculated by a specialized software with artificial intelligence

from KML Vision, Graz, Austria (IKOSA AI software). Three independent experimental series ($n=3$) were conducted.

Examination of anti-inflammatory potential

By addition of 1.5% dimethylsulfoxide to the culture medium, human promyelocytes were differentiated over a period of 6 days into functional neutrophils, which are capable to generate superoxide anion radicals after stimulation by a phorbol ester *in vitro* [16-18].

For the examination of endogenous radical formation, the differentiated cells were collected and washed several times with phosphate-buffered saline by centrifugation at 190 x g. Then, the functional neutrophils were added to a reaction mixture containing various concentrations of the fermentation drink, phosphate-buffered saline with 10 mM glucose, phorbol-12-myristat-13-acetate (Sigma-Aldrich, Deisenhofen, Germany) and the tetrazolium dye WST-1. The cleavage of the dye and the resulting color change was directly related to the amount of oxygen radicals present in the reaction mixture. The optical density was recorded at definite time points with the Elisareader (BioTek ELx808 with software Gen 5 version 3.00) up to 40 min and analyzed using Microsoft Excel. In order to calculate the extent of the oxidative burst, also the basal cell metabolism of the cells without stimulation by the phorbol ester was recorded simultaneously. Three independent experiments with triplicate wells were conducted ($n=3$).

STATISTICAL ANALYSIS

Statistical analysis of data was done using the two-tailed Wilcoxon-Mann-Whitney rank sum test. Significance was assumed at $p \leq 0.01$.

RESULTS

Antioxidant effect

As depicted in Figure 1, the antioxidant effect of EMIKO Vital increased in a dose-dependent manner with increasing concentrations and reached its maximum at the highest test concentration of 20 mg/ml with an inactivation of free radicals of more than 80%. Compared to the untreated control, this antioxidant effect was already statistically significant at the lowest EMIKO Vital concentration of 1 mg/ml ($p \leq 0.01$; Wilcoxon-Mann-Whitney test).

Oxidative stress

Since 0.25 mM hydrogen peroxide added to the culture medium did not cause any signs of cell death, only the higher concentrations of hydrogen peroxide up to 10 mg/ml were analyzed. The data showed that exogenous oxidative stress

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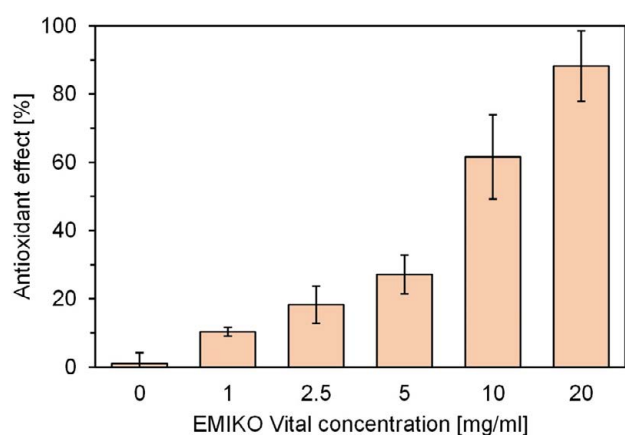


Figure 1: Graphical presentation of the dose-dependent inactivation of free exogenous radicals by the fermentation drink EMIKO Vital. Data represent mean value $\pm$ standard deviation of four independent experiments.

without protection by EMIKO Vital in the corresponding control group resulted in a cell viability of only $43.7 \pm 4.6\%$ (mean value $\pm$ standard deviation), while the cell viability with protection by EMIKO Vital was $85.3 \pm 8.9\%$ (mean value $\pm$ standard deviation). Therefore, the viability of intestinal epithelial cells was nearly doubled with the fermentation drink compared to the untreated control. The difference between the two cell groups was statistically significant ($p \leq 0.01$; Wilcoxon-Mann-Whitney test).

Cell regeneration/wound healing

As shown in Figure 2, EMIKO Vital significantly improved the regeneration of intestinal epithelial cells, i.e. that the residual cell-free area in the cell cultures treated with EMIKO Vital was significantly smaller than in control cells without EMIKO Vital. Remarkably, the fermentation drink resulted in a dose-dependent promotion of regeneration up to a test concentration of 10 mg/ml (= uncolonized residual cell-free area was lower), after which the regeneration process decreased (Figure 3). The maximum stimulation of regeneration was at 10 mg/ml. At the EMIKO Vital concentrations of 5 mg/ml and 10 mg/ml, the difference to the controls was statistically significant ($p \leq 0.05$ and $p \leq 0.01$; Wilcoxon-Mann-Whitney test).

Anti-inflammatory potential

The addition of EMIKO Vital caused a dose-dependent inhibition of endogenous radical generation by inflammation-mediating cells (Figure 4). From a concentration of 5 mg/ml, this inhibition was statistically significant ($p \leq 0.01$; Wilcoxon-Mann-Whitney test) and ranged from 25% at 5 mg/ml to almost 50% at 20 mg/ml.

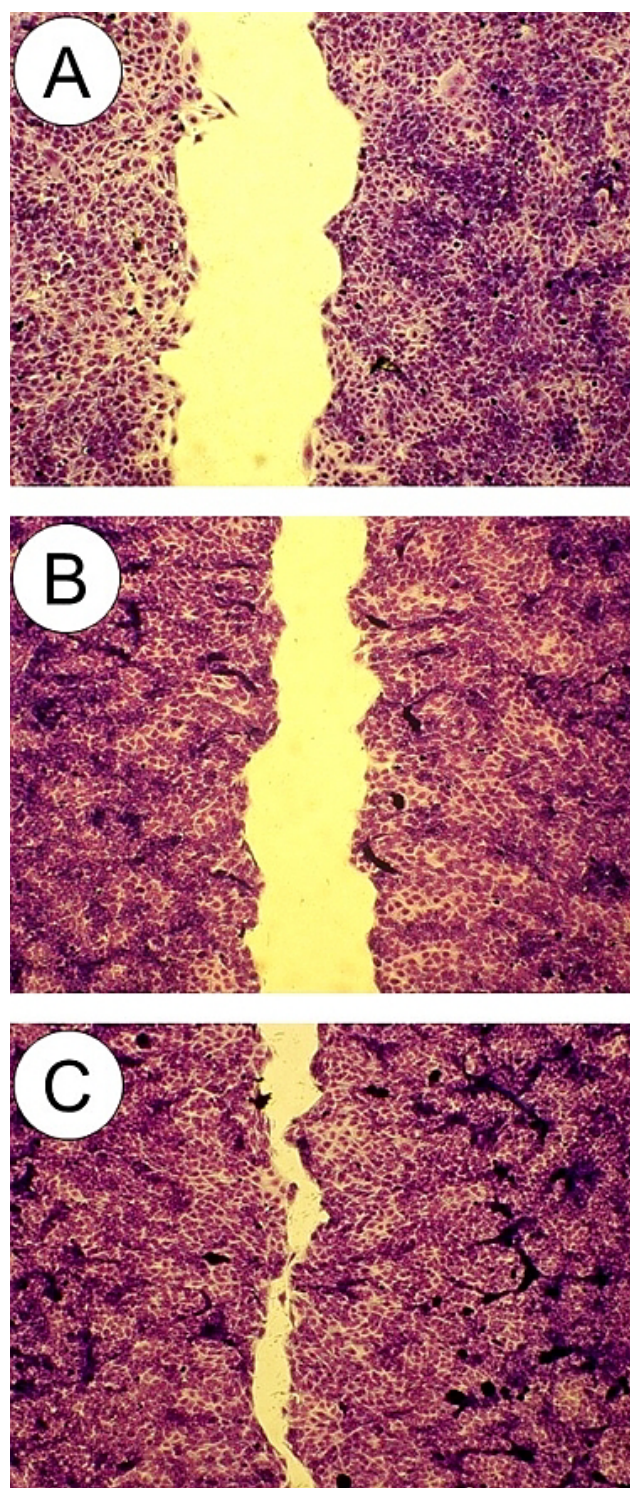


Figure 2: Representative micrographs of the fixed and stained intestinal epithelial cell cultures after 10 hours of regeneration. (A) Cell cultures without treatment = control, (B) cell cultures treated with 1 mg/ml of EMIKO Vital, (C) cell cultures treated with 10 mg/ml of EMIKO Vital. The difference in the colonization of the cell-free space is clearly visible. Olympus IX 50 inverted microscope at brightfield illumination with an Olympus Planachromate 10x and an Olympus E-20 digital camera at 5 megapixel resolution.

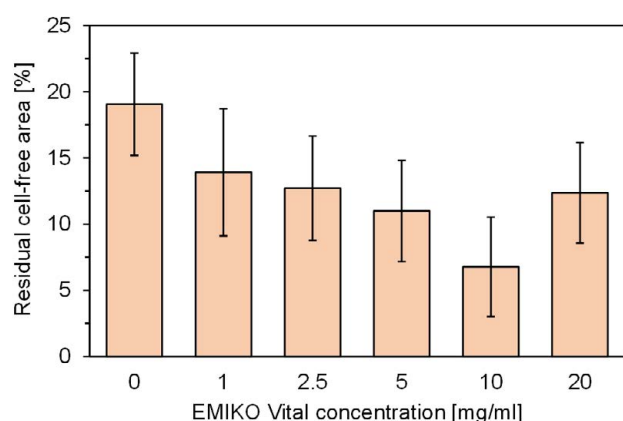


Figure 3: Graphical presentation of the uncolonized residual cell-free area at various EMIKO Vital concentrations. The uncolonized area is lowest at 10 mg/ml. Data represent mean value $\pm$ standard deviation of three independent experiments.

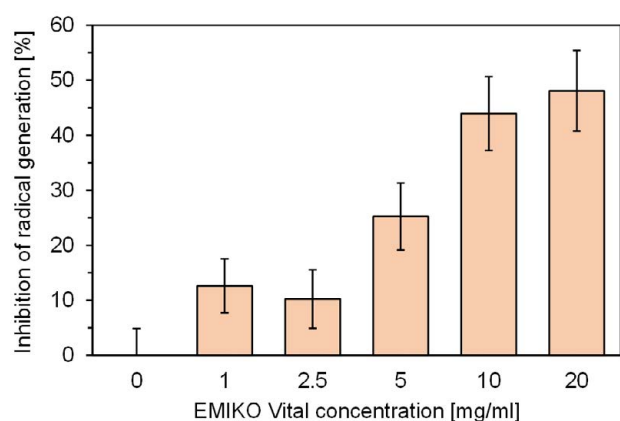


Figure 4: Graphical presentation of the dose-dependent inactivation of superoxide anion radicals generated by inflammation-mediating cells (functional neutrophils) by the fermentation drink EMIKO Vital. Data represent mean value $\pm$ standard deviation of three independent experiments.

DISCUSSION

Fermentation, a traditional method originally used for food preservation and storage, has evolved over time with advances in technology and a deeper understanding of the process. Today, fermentation is mainly used to improve food quality, enhance flavour and increase nutritional value [7,8]. The consumption of fermented foods has long been associated with health benefits. Many of these claims were based on anecdotal evidence, but more recently food and nutrition scientists have begun to provide more robust evidence for the beneficial effects of fermented products in health and disease [19]. Moreover,

fermented foods also provide a unique combination of microorganisms and bioactive compounds that can contribute to gastrointestinal health [20-23].

In order to investigate the beneficial intestinal effects of the fermentation drink EMIKO Vital, the IPEC-J2 cell line was chosen as the primary cell type, because “the IPEC-J2 cell line is unique as it is derived from the small intestine and is neither transformed nor tumorigenic in nature. IPEC-J2 cells mimic the human physiology more closely than any other cell line of non-human origin” [24]. The cells were originally isolated in 1989 by Helen Berschneider at the University of North Carolina [25]. The advantage of the IPEC-J2 cell line as an *in vitro* model originates from its morphological and functional similarities with intestinal epithelial cells *in vivo* [26]. The epithelial cells of the intestinal barrier have a high turnover rate, because they are quite sensitive against alterations of their environmental conditions involving a deficiency of the epithelium and immune/inflammation mediating cells [27].

As shown in this study, the fermentation drink EMIKO Vital has a basically high potential for the inactivation of reactive oxygen species. The result show no difference in efficacy between the inactivation of free exogenous radicals from the cellular environment and for radicals generated endogenously by an oxidative burst of inflammation-mediating cells (= functional neutrophils). In both cases, the dose-dependent mode of action is remarkable. However, the inactivation of exogenous free radicals is much more pronounced than that of endogenously generated radicals. This is a very important result, because the innate immune response in the blood by neutrophils should not be markedly influenced [28-31]. The promotion of cell regeneration/wound healing by EMIKO Vital is in line with its radical-inactivating effect. A quick regeneration is particularly important following intestinal injury caused by food intolerance, food additives and many other factors that can cause oxidative stress and consequently reduce intestinal permeability and function. Defects in intestinal barrier function play a pathogenic role in intestinal dysfunction and disease [32-34]. As reviewed by Farhadi et al. [2], a role for reactive oxygen species “in gastrointestinal-related abnormalities has been established for several gastrointestinal disorders. These include ischemic injury of the gastrointestinal mucosa, inflammatory bowel disease, peptic ulcer disease...” and others.

Taken together, the fermentation drink EMIKO Vital significantly compensates local oxidative stress and the associated consequences such as cell damage, delayed regeneration/healing, inflammatory processes and non-specific irritation of the intestine, thereby also improving systemic health.

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