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DETERMINATION OF THE ANTIOXIDANT ACTIVITY IN YOGURT

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Abstract

The highest risk from oxidative metabolism by-products is the formation free radicals. The damage to the organism caused by free radicals is immense and is a major threat for the welfare of the whole organism and is known to cause a variety of potentially fatal diseases.

The objective of this research is to determine the antioxidative capacity of fermented milk product (yogurt) with different types of microbiological strains. The method was spectrophotometric, used to determine antioxidant activity and the ability to neutralize the free 2,2-diphenyl-1-picrylhydrazyl radical.

Sterilized milk with 3.2% milk fat was fermented with different microbiological cultures including symbiotic starter culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus,* and monocultures *Lactobacillus acidophilus, L. casei* and *Bifidobacterium bifidus.*

All microbiological cultures showed a strong influence in increasing antioxidant activity of fermented product compared with the same value in unfermented milk. The highest antioxidant activity shows that milk fermented by *Lactobacillus acidophilus,* has measured 54.86% neutralization of free radical. The lowest value indicates milk fermented with symbiosis *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, measured 45.17%.

From this research can be concluded that the fermentation of milk is not a simple procedure, and does much more than simply extending shelf life of milk. It is a complex reaction which results in releasing different products such as peptides, free amino acids, enzymes and many different compounds which demonstrate an antioxidative capacity, and have role in maintaining the redox balance in the living organism.

***Key words**: Oxidative metabolism, Free radicals, Antioxidant activity, Fermented milk, Microbiological cultures.*

1. Introduction

The occurrence of byproducts of the different reactive oxygen species (ROS) in the aerobic organisms is a normal process. As an example of this ROS are the superoxide, hydroxyl, peroxyl and alkoxy radicals (Apel and Hirt [1]). In normal conditions in the organism, the processes of formation and neutralization or scavenging of the ROS are in balance. Different factors from the environment such as pollution, temperature and deficiency of nutrients, on a large scale can be correlated in the production of ROS. Also the oxidative stress in the organism is connected with the production of these free radicals. The high reactive potential of free radicals, and therefore the oxidative stress, is held for responsible in inducing some potentially fatal diseases such as carcinoma on different tissues and organs (Hallwell [2]), cardiovascular diseases, Parkinson's and Alzheimer's disease (Valko *et al*. [3]), atherosclerosis, necrosis of the myocard and induce degenerative processes of the myocard (Singh *et al*. [5]). Free radicals can also induce oxidative damage of proteins, DNA, and lipids in the organism especially on the lipids of the cellular wall therefore causing cellular death (Jacobi and Burri, [4]).

The oxidative stress reflects the intracellular balance between the systematic manifestations of the ROS and the ability of the biological system to detoxify these reactive byproducts or to repair the damage occurred by their action. The disruption of the normal redox status of the cells can cause toxic effects trough production of peroxides and free radicals which can damage all cellular components including proteins lipids and DNA. Because some ROS play an important role in cellular signaling, some types of ROS and therefore the oxidative stress can disrupt the natural process of cellular communication which results in cellular overload with ROS and death of the cell (Hallwell [2]).

From chemical aspect, the oxidative stress is associated with increased production of oxidative species or/ and significant decrease of the efficacy and efficiency

of the intracellular antioxidative defense mechanisms such as glutathione (GSH), superoxide dismutase (SOD) etc (Schafer and Buettner [6]). The effects of oxidative stress are dependent on the scale of the changes in the cell because the defense mechanisms in the cell are able to repair the damage and to establish a redox balance and to regain its primary state. On the other hand, a more intense oxidative stress can cause cellular death and necrosis of local tissue and moderate oxidative stress can cause premature apoptosis (Lennon *et al*. [7]).

Milk proteins are an abundant source of bioactive peptides. Milk and especially its fermented products in recent time are gaining in popularity as an component in health beneficial functional types of food, which can have a preventative effect on some modern age diseases caused by improper diet such as cardiovascular diseases, diabetes type II and obesity. These peptides are inert in the initial protein of the milk (casein and albumins), and can be activated and released into the medium by several processes such as enzymatic hydrolysis of the protein by digestive enzymes, fermentation of the milk by various microbiological strains and proteolysis by vegetative or microbiological enzymes in cheese production (Hannu, [8]).

The fermentation of the milk with lactic acid bacteria results in delivering a vast number of bioactive peptides and free amino acids with different biological activity such as inhibition of the angiotensin converting enzyme (Nielsen *et al*. [9]; Tomovska *et al*. [10], [11]), immune activity (Coste *et al*. [12]) and antioxidative activity (Pena-Ramos *et al*. [13]).

Antioxidant vitamins in milk have a great contribution in the daily intake of these nutrients. Vitamin E and carotenoids, for example, are located in the membranes of the fat globules in milk where they prevent auto oxidation of the milk fat. Vitamin C is an important antioxidant which has a complex interaction with the iron and is an electron donor in the conversion of the tocopheroxyl radical back to antioxidant active vitamin E (Lindmark and Akesson [14]).

Lactoferrin is assumed to have an important role in chelating iron ions, thus preventing the oxidation of fatty acids and releasing peroxy radicals. The lactoferrin is a protein with molecular mass of 80 kDa, and can be found in milk whey from different species. In cow's milk lactoferrin is present with concentrations of 20 - 200 mg/L dependent of animal health, age and nutrition. To the lactoferrin are attributed several functions such as increase of iron absorption in the organism, bacteriostatic and bactericide function, growth factor and a potent antioxidant (Bihel and Birlouez-Aragon [15]).

The concentration of these antioxidants in milk is I great correlation with the nutrition of the dairy cattle and on the manner the milk is stored. Because the milk is abundant with antioxidants and every antioxidant has a specific reaction the exact function of each antioxidant in milk cannot be determined. Because many components of the milk present themselves with a certain antioxidant capacity, a measurement of the total antioxidant capacity can be a useful method for determining the sum of the antioxidant role of every component of the milk (Lindmark and Akesson [14]).

The objective in this research was to determine the total antioxidant capacity of fermented dairy products using different strains of microorganisms. For that manner symbiotic starter cultures and monocultures were used.

2. Materials and Methods

As a medium for this research, sterilized whole cow's milk was used. The milk showed the average chemical structure shown in Table 1.

Table 1: Average chemical composition of milk

Components of milk	Quantity
Protein	3,1g
Milk fat	3,2g
Lactose	4..5g
Calcium	120 mg/mL

The milk was fermented with different starter cultures:

- Symbiosis of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus;*
- Monoculture - *Lactobacillus casei*;
- Monoculture - *Lactobacillus acidophilus* and
- Monoculture - *Bifidobacterium bufidus*.

All cultures were purchased from CHR-Hansen, Denmark. The fermentation was carried out according to industrial standards and by the instructions provided by the manufacturer for each culture.

Preparation of fermented milk

Before fermentation the milk was heated to 35 ^{0}C in a sterile container. The inoculation with the cultures in direct, according to industrial standard of 0.01% w/v. After inoculation the milk was stirred with a sterile whisk for 5 min. to disperse the microbiological cultures evenly. Before opening the bad containing the cultures, the edge of the bag and the scissors were sterilized with 96% v/v ethanol.

The fermentation was carried out in single use sterile cups, and in an incubator Elecrem Y-140 at 40 ^{0}C. The time needed for fermentation was 4 hours for the cultures in symbiosis *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus,* and 12 hours for

the rest cultures, or until a pH value of 4,6 was reached. Immediately after the fermentation the cups were stored at 4 ^{0}C for the whole duration of the research. The measurement of the total antioxidant capacity was done at several intervals which were: immediately after fermentation, 1st, 2nd, 3rd, 5th, 10th and fifteenth day after fermentation.

Determination of the antioxidant activity of fermented milk

Preparation of whey fraction from fermented milk

The whey fraction was prepared essentially as described by Virtanen *et al*. [16] and used immediately after the preparation. Non-hydrolyzed casein was removed. Aliquots (15 mL) were collected from the fermented milk and the pH was adjusted (if needed after fermentation) to 4.6 with 1 M HCl. The suspension was centrifuged (9000rpm^{-1}, RCF = 7690g for 20 min at 5 ^{0}C in a Hettich Universal 320R, Andreas Hettich GmbH – Germany, and the supernatant was filtered on a 0.45-mm filter (Millipore Corp, Billerica, MA, USA).

DPPH radical scavenging activity

DPPH radical scavenging activity was evaluated using the method of Son and Lewis [17]. DPPH was used as a stable radical. A volume of 2 mL of DPPH in ethanol (500 mM) was added to 2 mL of the whey fraction, mixed vigorously and allowed to stand in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. Ethanol was used as a blank, while DPPH solution in ethanol served as the control. The radical scavenging activity of the samples was expressed as % inhibition of DPPH absorbance:

$$\text{Inhibition} = [(A_{control} - A_{test})/A_{control}] \times 100$$

Where:

- *Acontrol* is the absorbance of the control sample (DPPH solution without whey fraction) and
- *Atest* is the absorbance of test sample (DPPH solution plus whey fraction).

3. Results and Discussion

Fermentation of milk represents a very beneficial method for prolonging shelf life and a great way to obtain so called functional foods with sought for acceptable flavor. The fermentation is important in producing dairy products, because the various strains ob microbiological cultures used degrade the primary components of milk such as protein, carbohydrate and lipids into various secondary forms like free amino acids, peptides, organic acids, free fatty acids, which may possess various health beneficial functions. In this manner, the free amino acids and peptide sequences possess antihypertensive effect (Tomovska *et al*. [11]), increasing of antioxidant capacity and inhibition of lipid peroxidation (Virtanen *et al*. [16]) which functions can be very beneficial for the human health and well-being.

In Table 2 are presented the results obtained for the antioxidant activity of milk fermented with various strains of microbiological cultures.

Table 2: Values for the antioxidant activity

Days	*L. bulgaricus + S. thermophilus*	*L. casei*	*L. acidophilus*	*B. bufidus*
0* **	6.13	6.13	6.13	6.13
0	52.44	56.51	63.99	54.93
1	45.43	49.39	58.77	52.13
3	39.43	49.06	55.67	46.46
5	43.01	47.61	49.48	44.03
10	43.34	52.87	48.60	42.26
15	47.42	54.55	52.64	45.31
$\bar{x}$ *	45.18	51.66	54.86	47.52
SD**	4.45	3.51	5.87	4.94

*Average values without initial values for antioxidant activity of sterilized milk
**Standard deviation without initial values for antioxidant activity of sterilized milk
*** Initial values for antioxidant activity of sterilized milk

The dynamics of the development of the antioxidant activity for the examined cultures is presented in Figure 3. From the diagram can be compared the antioxidant activity of all cultures during the whole period of the research and the changes made to the initial input-unfermented milk.

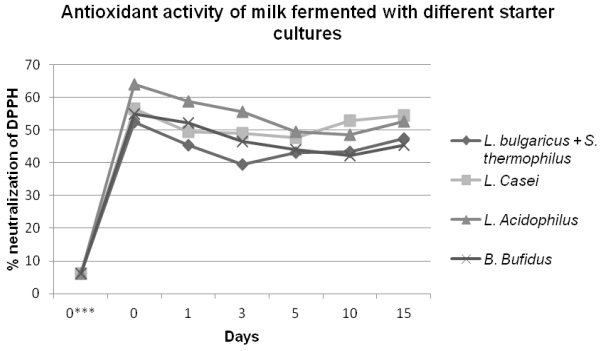

Figure 3. Values for the antioxidant activity

From the diagram can be concluded drastic increase of the antioxidant activity for each culture compared to the unfermented milk. Also a linear trend can be established for the antioxidant activity, with minor deviations in the control points.

Overall, highest average value for the antioxidant activity presents the milk fermented with *Lactobacillus acidophilus* (54.86%) and the lowest value the milk fermented with the symbiotic cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (45.18%).

The milk fermented with the symbiotic cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* records its highest value for the antioxidant activity right after the fermentation has finished and has a value of 52.44%. The lowest value for the antioxidant activity of the same cultures is recorded at the 3rd day of storage at 4 ^{0}C with a value of 39.43%.

The milk fermented with *Lactobacillus casei* also records its highest value for the antioxidant activity right after the fermentation has finished and has a value of 56.51%. The lowest value for the antioxidant activity of the same culture is recorded at the 5th day of storage at 4 ^{0}C with a value of 47.61% neutralization of DPPH free radical.

The milk fermented with *Lactobacillus acidophilus* also records its highest value for the antioxidant activity right after the fermentation has finished and has a value of 63.99%. The lowest value for the antioxidant activity of the same culture is recorded at the 10th day of storage at 4 ^{0}C with a value of 48.60% neutralization of DPPH free radical.

The milk fermented with *Bifidobacterium bifidus* also records its highest value for the antioxidant activity right after the fermentation has finished and has a value of 54.93%. The lowest value for the antioxidant activity of the same culture is recorded at the 10th day of storage at 4 ^{0}C with a value of 42.26% neutralization of DPPH free radical.

As a reference, Vitranen *et al*. (2007) using the ABTS free radical method has concluded that the antioxidant activity of *Lactobacillus acidophilus* is 42%, while in *Lactobacillus casei* is 4%. Also these authors have studied the antioxidant activity of the symbiosis between *Lactobacillus acidophilus* and *Bifidobacterium infantis* and have measured an average of 27% neutralization of ABTS free radical. This point out to a lowering of the antioxidant activity of the fermented milk with the symbiosis *Lactobacillus acidophilus* and *Bifidobacterium infantis* compared with the same value in the monoculture *Lactobacillus acidophilus*.

The difference of the values for the antioxidant activity may also be dependent on the conditions in the laboratory during the trial, such as conditions under which the fermentation takes place, sensitivity of instruments used and the sanitary conditions in the laboratory.

4. Conclusions

From the conducted research for antioxidant activity of fermented milk, the following can be concluded:

- The fermentation of milk does something more than a simple prolonging of shelf life. The fermentation makes some molecular changes in the milk which results in releasing different compounds such as peptides, free amino and fatty acids which possess antioxidant capacity.

- The fermentation of the milk results in boosting the antioxidant effect in the dairy product. This effect is achieved with every microbiological culture tested in this research.

- The highest value of antioxidant capacity has the milk fermented with the probiotic strain *Lactobacillus acidophilus* and this activity was at its peak right after fermentation and the value was 63.99% neutralization of DPPH free radical.

- The lowest antioxidant activity possesses the milk fermented with the symbiotic cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. This value was measured at the third day and was 39.43% neutralization of DPPH free radical.

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