IMPROVE THE NUTRITIONAL VALUE OF FERMENTED DAIRY PRODUCTS BY NUTRACEUTICAL-PRODUCING FOOD-GRADE MICROORGANISMS

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Abstract

Over past five years the range of dairy products enriched with added vitamins has grown dramatically. The consumers apply for vitamin enriched dairy products. Food plus refers to category of value-added products that provide health benefits beyond basic nutrition through the inclusion of new or increased levels of key ingredients.
The study is a part of the „Nutra Cells" large European research project which is investigated the possibility of the vitamin B_{12} production by the new strain of ***Propionibacteria freudenreichii ssp. shermanii.*** *The company where the research was realized have been quick to recognize the importance of the fortified dairy products and are also trying the new ways to obtain increased level of vitamins in dairy products.*
The samples of yoghurt incorporated in different concentration 1% (v/v) and 5% (v/v) and different medium Lactate and Milk preculture with Propionibacterium freudenreichii ssp. shermanii B 374-2, was tasted and compared with BY 330 (reference yoghurt), basic Lactate and Milk preculture and Milk. The samples were measured at the beginning of fermentation (t = 0), in the end (t = 4,5 pH) and after 14 days of storage at 4°C in two independent yoghurt trials.The presence and the growth of Propionibacterium freudenreichii ssp. shermanii B 374-2,2 during the fermentation and after storage at 4°C was monitored by counting of CFU´s in Lactate medium in duplicate measurements. Vitamin B_{12} was determined by a Microbiological Bio-assay with indicator strain Lactobacillus delbrueckii ssp. leichmanii.
In conclusion, it is possible to say that the new strain of ***Propionibacterium freudenreichii ssp. shermanii*** *was proved like a very good producer of vitamin B_{12} during yoghurt fermentation and storage at 4°C and therefore it can be used to fortify dairy products.*

Key words: fermented dairy products, healthful food, Nutraceuticals, *Propionibacterium freudenreichii ssp. shermanii,* Vitamin B_{12}, Microbiological Bio-assay.

INTRODUCTION

According to the literature, Propionibacteria are well known as producer of vitamin B_{12} . The aim of the research is to improve the nutritional value of fermented dairy products by the nutraceutical-producing food grade microorganisms due to increasing the vitamin B_{12} level in yoghurt products by Propionibacteria.
The new strain *Propionibacterium freudenreichii ssp. shermanii* was tested here as a producer of vitamin B_{12}.
Milk is the most nutritionally complete food containing nearly all the constituents of nutritional importance to humans. Milk is excellent source of most B vitamins[2, 10]. Yoghurt is made by fermentation milk of two types of lactic acid bacteria, namely ***Streptococcus thermophilus*** and ***Lactobacillus bulgaricus.*** Yoghurt like a healthful food is a great source of protein and calcium. Yoghurt is a unique dairy food because the starter cultures actually produce enzyme which degrade a lactose sugar during fermentation. Yoghurt starter cultures are using for their possible role in just about everything from improved digestion and reduced risk of intestinal infection to improved immune function and reduced risk of certain cancers[1].
The term vitamin originated from "vitamine," a word first used in 1911 to designate a group of compounds considered vital for life. Vitamins are any of several organic substances that are essential in human nutrition. Usually are separated into water-soluble (the B vitamins, vitamin C) and fat-soluble (vitamins A, D, E, K) groups. Vitamins play a huge role in nearly all of the basic functions of the body. Vitamins are used in application sectors: supplements, food enrichment and increasingly in cosmetics. Vitamin B_{12} came to prominence in the scientific world in 1920. The interest in the development of vitamin B_{12} reached a peak in the 1960s and then decreased slowly[6, 8]. From the chemical point of view, the term vitamin B_{12} is synonymous with cyanocobalamin. Is also usually called cobalamin. Vitamin B_{12} is a water soluble, unstable to light and temperature, an essencial vitamin in the human diet. The cobalamins crystallize in red nedles and are present in nature in their coenzyme form. The group of cobalamins includes cyanocobalamin, hydroxocobalamin, methylcobalamin and 5'-desoxyadenosilcobalamin. Other cobaltcorrinoids have different heterocyclic bases (like substituted benzimidazoles or purines)[8]. Humans can not synthetize this vitamin and that is the reason why must be obtain it from organisms that produce it. Animal tissues that accumulate vitamin B_{12} are excellent food sources of the vitamin. It is naturally found in animal

foods including fish, milk and milk products, eggs, meat, and poultry [3].

The recommended dairy intake (RDI) of this vitamin is 1-3 µg per day. Characteristic signs of B_{12} deficiency include fatigue, weakness, nausea, constipation, flatulence (gas), loss of appetite, and weight loss. Deficiency also can lead to neurological changes such as numbness and tingling in the hands and feet. Additional symptoms of vitamin B_{12} deficiency are difficulty in maintaining balance, depression, confusion, poor memory, and soreness of the mouth or tongue. Malnutrition also can lead to most notably anemia.[4].

Propionibacteria are a genus of gram positive, facultatively anaerobic, nonmotile, non-spore-forming, rod shaped (bacillus) bacteria, found as saprophytes in humans, animals and dairy products. Propionibacteria play an important role in several industrial processes, have been more widely used for the production of starters for the Swiss-type cheese. Other industrial applicants are probiotics production, vitamin B_{12} and propionic acid production by fermentation of glucose. *Pro*biotics are complex sugars (such as inulin and other fructo-oligosaccharides) that are ingested as fuel for bacteria already present in the gastrointestinal tract. When the normal balance of these bacteria is disturbed by illness or antibiotic treatment, the most common effect is diarrhea. Probiotics work by colonizing the small intestine and crowding out disease-causing bacteria, thereby restoring balance to the intestinal flora [3].

The genus *Propionibacterium* can be divided into two groups, a group containing the classical (or dairy) propionibacteria and a group containing the cutaneous propionibacteria [9]. Classical propionibacteria, nonpathogenic organisms, presented in cheese and milk are classified into six species: *P. freudenreichii, P. jensenii, P. theonii, P. acidipropionici, P. coccoides* and *P. cyclohexanicum.* The largest cluster , representing 37% of the strains, is P. jensenii.

Cutaneous propionibacteria are also known as anaerobic coryneforms or anaerobic diphtheroides. The main habitat of cutaneous propionibacteria is humam skin and are classified into six species too: *P. acnes, P. avidium, P. granulosum, P. lymphophilums, P. propionicum* and *Propioniferax innocua* [5].

MATERIALS AND METHODS

The amount of the Propionibacteria is determined as colony forming units (CFU). The yoghurt samples are diluted in Ringer solution untill the appointed dilutions (estimation of 100 CFU´s per ml). 100 µl of the dilutions are plated on suitable medium. Propionibacteria grow on Lacteta broth.

The quantification of the vitamin B_{12} content in food using the microbiological assay is based on the determination of the optical density at 546 nm. This method is working by the fact that the growth of Lactobacillus delbrueckii ssp. leichmanii (ATCC 7830) depends on the concentration of vitamin B_{12} available in food. For use the stock of this culture is defrosted and inoculated in a sterile vitamin B_{12} free assay medium.

In this study were used like a incubation medium of Propionibacteria grow the pre-preculture of Propionibacteria, milk preculture and lactate preculture. Pre-preculture is necessary to obtain milk preculture and preculture of Lactate.

The yoghurt preparation is made of semi-skimmed retail milk with addition of yoghurt culture concentrate BY 330, milk preculture and lactate preculture. This yoghurt is fermented at 30°C for co-cultivation of Propionibacteria.

These samples are carefully mixed and plated on Lactate agar in laminar flow cabinet (t = 0). From each bottle 10 ml is put in a parallel-tube to monitor pH (t = 0) and during the acidification of yoghurt also.

After then the yoghurt samples are inserted in waterbath or incubator with 30°C until pH 4,5 (t = pH 4,5). The samples are storage in 4°C for 14 days to determinate the CFU´s per ml (t = 14 days). Yoghurt samples are sensorial tested.

The samples are diluted 1: 10 (v/v) with a KCN-solution and filled into the centrifuge tubes. After homogenization the samples are boiled at 98°C for 30 minutes. Then the samples are centrifuged and the supernatant is used for the Bio-assay.

A dilution range is prepared for the reference line as well as for samples. Tubes are covered and sterilized at 121°C for 15 minutes. The cooled tubes are inoculated with defrosted Lactobacillus delbrueckii ssp. leichmanii. After all tubes are incubated for at least 24 hour at 37°C and are measured spectrometric against the blank at 546 nm.

The vitamin B_{12} amount is determined by measuring the growth of the indicator strain. The vitamin B_{12} content is calculated in µg cyanocobalamin per 100ml by using the standard curve.

RESULTS AND DISCUSSION

Sensory test: The best time for tasting yoghurt is after 14 days of storage at 4°C. It was tasted five samples in total. The yoghurt samples were tasted twice. The taste was compared to reference yoghurt BY 330. Satisfactory results in tasting show samples 5% B 374-2,2 milk and 1% B 374-2,2 lactate.

CFU´s of Propionibacteria in milk by way of Yoghurt test: In experiment of Yoghurt test there is no growth of the Propionibacterium freudenreichii ssp. shermanii B 374-2,2 by reference yoghurt BY 330. That are a right results because there aren´t inoculated Propionibacteria B 374 – 2,2. The percentage confrontation of 1% (v/v) and 5% (v/v) samples are in accordance with ours expectation. Five percent has quintuple content than one percent.

During the fermentation and storage of yoghurt samples incorporated with Propionibacterium B 374- 2,2 the amount of CFU ´s stay in the same level in all cases. We can see here no increase and no decrease of CFU during storage. This is different effect in comparison with measurements of vitamin B_{12} content Bio-assay.

Vitamin B_{12} content measured by the Microbiological Bio-assay: In experiment of Microbiological Vitamin B_{12} Bio-assay Method the highest values of concentration vitamin B_{12} - around the 1,1µg/100ml show real Lactate broth incorporated with the Propionibacterium freudenreichii ssp. shermanii B 374-2,2 like a Milk incorporated with the Propionibacterium freudenreichii ssp. shermanii B 374-2,2 and 5% casiton. Normal milk contains content of vitamin B_{12} of around 0,6 µg/100ml. Milk has in all measurements lower values in comparison with co-cultivated yoghurts.

In the samples of reference yoghurt BY 330 it is possible to see initial values around 0,75 µg/100ml and then decreasing values during the fermentation in all cases of BY 330 samples. So the vitamin B_{12} content in the reference yoghurt BY 330 decline. The decreasing of BY 330 during the time is considerable than by others co-cultivated yoghurts. The decrease of vitamin B_{12} is attributed to consumption by starter cultures Lactic acid bacteria during storage, because there is no heat treatment after the fermentation and the Lactic acid bacteria are remain viable [0].

In all co-cultivated yoghurts with the Propionibacterium freudenreichii ssp. shermanii B 374-2,2 there is evident increasing level of vitamin B_{12} content by samples t = 4,5 pH and t = 14 days. In results is sometimes possible to see decreasing of vitamin B_{12} content during the fermentation and during the 14 days of storage.

In all co-cultivated yoghurts with the Propionibacterium freudenreichii ssp. shermanii B 374-2,2 is possible to see a higher amount of vitamin B_{12} in comparison with reference yoghurt BY 330.

CONCLUSIONS

During the fermentation and storage of the Yoghurt test the amount of CFU 's incorporated with Propionibacterium B 374- 2,2 stay in the same level in all cases. We can see here no increase and no decrease of CFU during storage.

In case of Microbiological Vitamin B_{12} Bio-assay Method the highest and comparable values with approximately the same concentration of vitamin B_{12} show the Milk preculture and the Lactate preculture. Their medium content is about 1,1 µg/100ml. The almost a half value has a concentration of milk 0,58 µg/100ml.

All co-cultivated yoghurts with the Propionibacterium freudenreichii ssp. shermanii B 374-2,2 show a higher amount of vitamin B_{12} after 14 days of storage in comparison with reference yoghurt BY 330. There is also evident decrease of vitamin B_{12} content from t = 0 to t = 14 days in nearly all yoghurts included the reference yoghurt BY 330.

This new strain ***Propionibacterium freudenreichii ssp. shermanii*** was proved like a very good producer of vitamin B_{12} and ***5% Propionibacterium freudenreichii ssp. shermanii*** is possible to recommended to fortification of fermented dairy products.

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Samples (ml)	1	2	3	4	5
BY 330 - concentrate	0,4	0,4	0,4	0,4	0,4
Milk preculture	/	2	10	/	/
Lactate preculture	/	/	/	2	10
Milk	200	200	200	200	200

Tab. 1. : The yoghurt preparation

Samples	
1	BY 330 (reference yoghurt)
2	1% B 374-2,2 milk
3	5% B 374-2,2 milk
4	1% B 374-2,2 lactate
5	5% B 374-2,2 lactate

Tab. 2. : Identification of samples

Samples	Sensory properties
1. BY 330	Dry, sour (fresh) taste, yoghurt consistence
2. 1% B 374-2,2 milk	Mild, sweet taste
3. 5% B 374-2,2 milk	Milder taste then 1% B 374 – 2,2, pleasant taste
4. 1% B 374-2,2 lactate	Pleasant taste, a little bit after a medium
5. 5% B 374-2,2 lactate	Intensive odour and taste after a medium

Tab. 3. : Characteristic of sensory test

Preculture	CFU per ml	pH
Milk preculture	$6,3 . 10^7$	5,94
Lactate preculture	$5,5 . 10^8$	5,64

Samples	t = 0	pH	t = 4,5 pH	pH	t = 14 d	pH
BY 330	/	6,5	/	4,49	/	4,27
1% B 374-2,2 milk	$5,0 . 10^5$	6,5	$1,1 . 10^6$	4,47	$5,0 . 10^5$	4,23
5 % B 374 – 2,2 milk	$1,1 . 10^7$	6,5	$8,3 . 10^6$	4,46	$2,5 . 10^6$	4,24
1% B 374-2,2 lactate	$3,5 . 10^6$	6,5	$3,5 . 10^6$	4,54	$1,5 . 10^6$	4,27
5 % B 374 – 2,2 lactate	$2,4. 10^7$	6,5	$1,4 . 10^7$	4,49	$2,0 . 10^7$	4,29

Tab. 4. : Results of the 1st yoghurt trial (Experiment A) and calculation of the produced vitamin B_{12} amount per CFU in yoghurt incorporated with Propionibacterium

Preculture	CFU per1.8 ml	pH
Milk preculture	$1,8 . 10^7$	6,35
Lactate preculture	$2 . 10^8$	5,89

Samples	t = 0	pH	t = 4,5 pH	pH	t = 14 d	pH
BY 330	/	6,62	/	4,11	/	4,15
1% Prop. milk	$5,0 . 10^5$	6,63	$1,8 . 10^6$	4,10	$6,5 . 10^5$	4,13
5 % Prop. milk	$3,0 . 10^6$	6,62	$4,5 . 10^6$	4,10	$2,0 . 10^6$	4,11
1% Prop. lactate	$4,8 . 10^6$	6,61	$1,6 . 10^7$	4,13	$9,3 . 10^6$	4,09
5 % Prop. lactate	$1,5 . 10^7$	6,58	$2,4 . 10^7$	4,21	$4,8 . 10^7$	4,24

Tab. 5. : Results of the 2nd yoghurt trial (Experiment B) and calculation of the produced vitamin B_{12} amount per CFU in yoghurt incorporated with Propionibacterium

c of vitamin B_{12} (mg/100ml)				
Samples	**t = 0**	**t = 4,5**	**t = 14 d**	**free time**
BY 330	0,76 ± 0,08	0,52 ± 0,04	0,40 ± 0,03	/
1% P. milk	0,70 ± 0,13	0,60 ± 0,06	0,50 ± 0,05	/
5% P. milk	0,75 ± 0,20	0,72 ± 0,07	0,61 ± 0,05	/
1% P. lactate	0,77 ± 0,07	0,65 ± 0,05	0,60 ± 0,04	/
5% P. lactate	0,72 ± 0,05	0,85 ± 0,08	0,79 ± 0,04	/
Lactate + P. (LP)	/	/	/	0,98 ± 0,22
Milk + cas + P. (MP)	/	/	/	1,02 ± 0,20
Milk	/	/	/	0,58 ± 0,09

Tab. 6. : Concentration of vitamin B_{12} and standard deviation

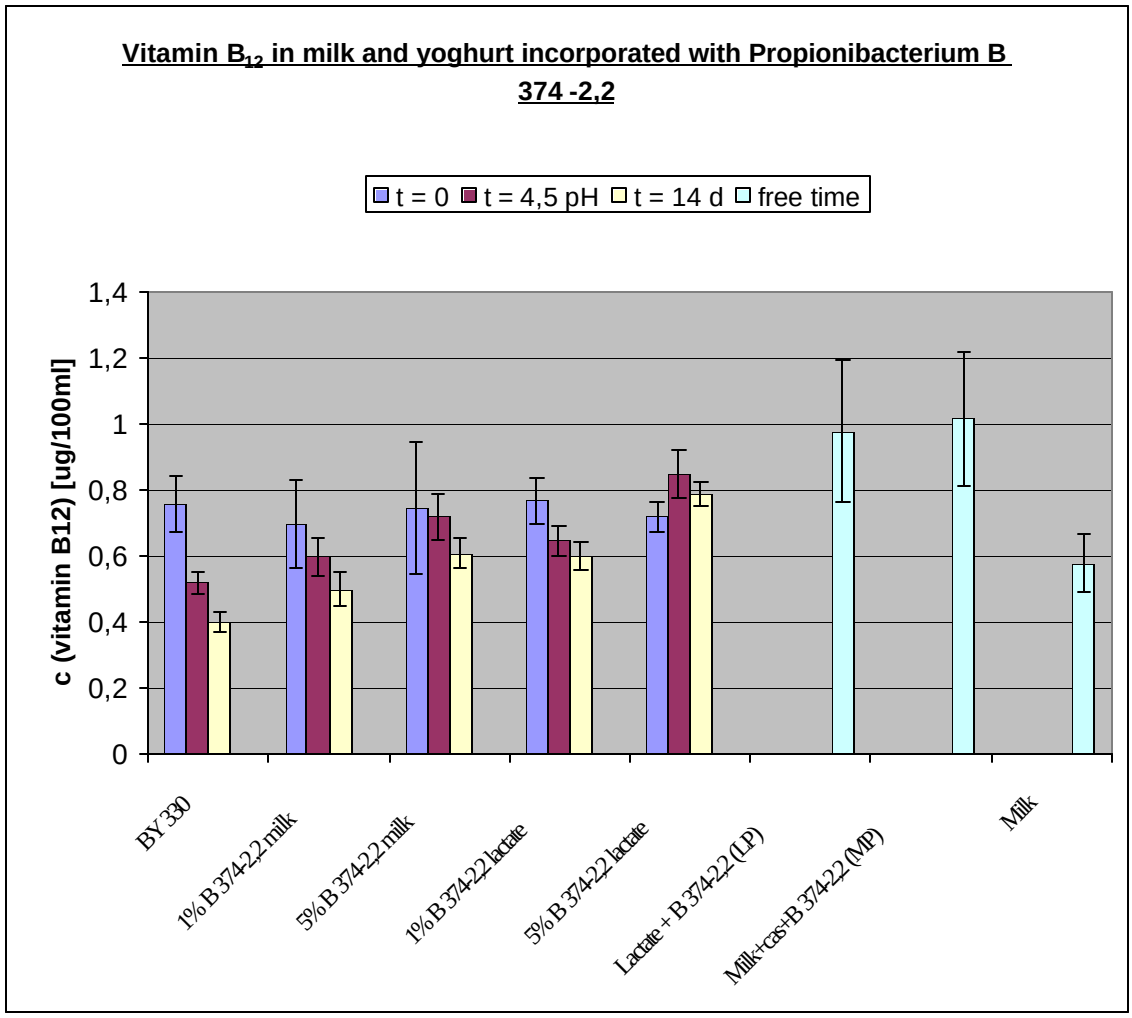

Fig. 1. : The mean results of two measurements with the same samples preparation (1st yoghurt trial) of vitamin B_{12} determination in milk and yoghurt incorporated with Propionibacterium
LP – Lactate preculture
MP – Milk preculture

c of vitamin B_{12} (mg/100ml)				
Samples	**t = 0**	**t = 4,5**	**t = 14 d**	**free time**
BY 330	0,81 ± 0,05	0,68 ±0,07	0,49 ± 0,14	/
1% P. milk	0,69 ± 0,08	0,76 ± 0,04	0,78 ± 0,13	/
5% P. milk	0,83 ± 0,07	0,79 ± 0,09	0,78 ± 0,05	/
1% P. lactate	0,73 ± 0,06	0,84 ± 0,11	0,84 ± 0,19	/
5% P. lactate	0,75 ± 0,11	0,93 ± 0,13	0,95 ± 0,23	/
Lactate + P. (LP)	/	/	/	1,16 ± 0,36
Milk + cas + P. (MP)	/	/	/	1,21 ± 0,14
M ilk	/	/	/	0,64 ± 0,08

Tab. 7. : Concentration of vitamin B_{12} and standard deviation

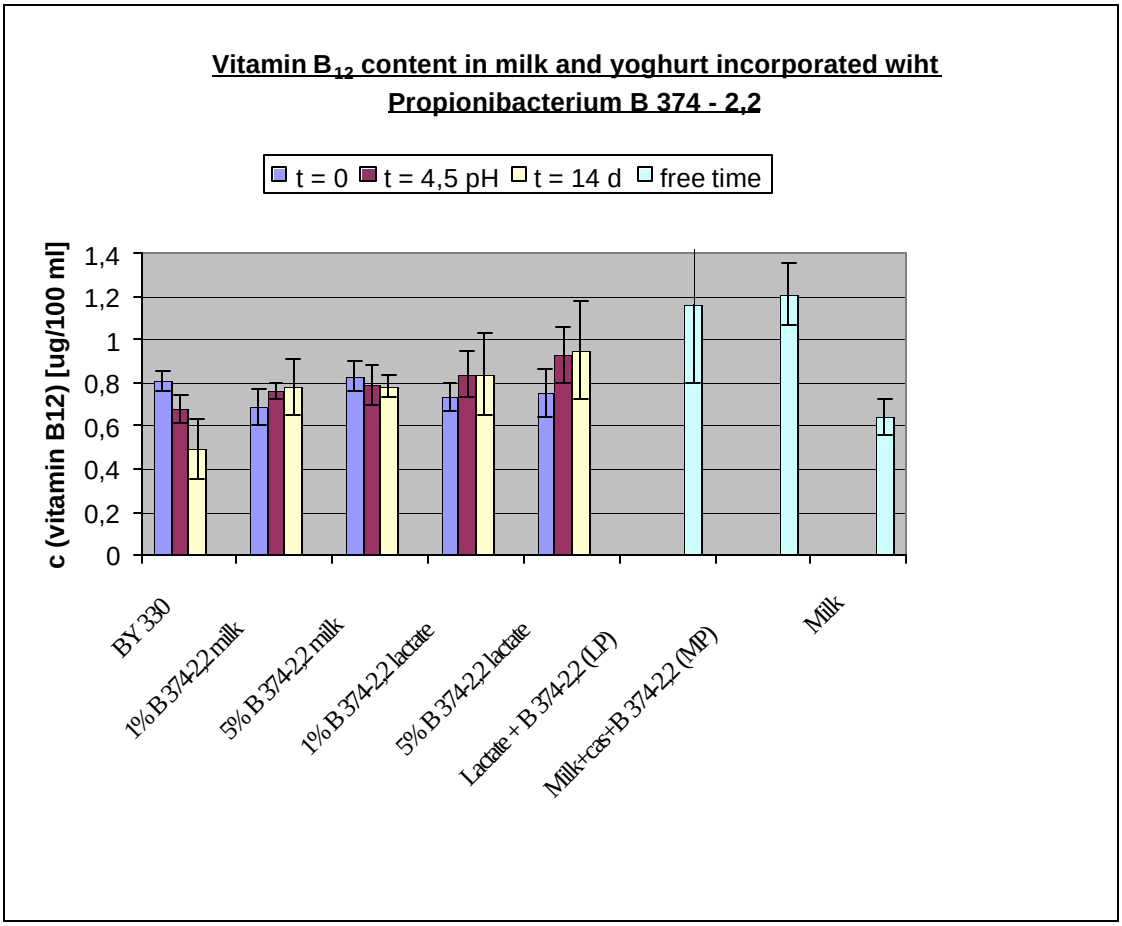

Fig. 2. : The mean results of two measurements with the same samples preparation (1st yoghurt trial) of vitamin B_{12} determination in milk and yoghurt incorporated with Propionibacterium