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APPLICATION OF ISOTHERM CALORIMETRY IN THE DEVELOPMENT OF FOODS CONTAINING PROBIOTIC LIVE FLORA AND ENRICHED WITH BIOAVAILABLE Ca^{2+}

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The development of functional foods of probiotic effect based on the slime-producing strains isolated in the 1980s, and that of enriched with Ca on the utilization of the high Ca-containing whey of the quarg production in the Carpathian basin using fermentation. The probiotic properties of the slime-producing microbe strains isolated have been proved by in vitro and in vivo examinations. We have used an isotherm DSC method to identify the probiotic microbes. The percentile ratio of probiotic and other microbes was determined in the product by this technique. By utilization of quarg whey a special additive food for Ca-enrichment has been developed which is suitable to complete or enrich different foods (dairy, meat and bakery products).

The products developed are: probiotic kefir (Synbiofir), probiotic sour cream, probiotic butter cream, poultry meat products completed with Ca, bakery products completed with Ca.

Keywords: *additive-food for Ca-enrichment, foods completed with Ca, isotherm calorimetry, probiotic dairy products, probiotic microbes*

Introduction

Research of the functional dairy products has at least thirty years history in Hungary. The human physiological effects of newly developed dairy products as well as other functional parameters of dairy products can be found in the book edited by the Hungarian Dairy Research Institute (HDRI) and the former University Medical School of Pécs (now Faculty of Medicine, University of Pécs) [1]. These works are the basis of the development and introducing butter creams protected by patent [2] which are nowadays the only traditional dairy products without any decrease in the consumption. The human physiological goodness of the individual microorganisms was realized at the beginning of the last century [3] the investigation of the probiotic products culminated only at the end of the last century. The HDRI during the years of 1979–1981 has isolated in the frame of an international cooperation a slime-producing strain; they could prove its probiotic characteristics only in the first years of 21st century [4].

The other big group of the functional dairy products is the dairy products enriched in Ca^{2+}. The main motivation of the development of these kinds of products was the dangerous propagation of osteoporosis disease. As a 'side result' of the investigation of this disease was a surprising fact: the Ca:P ratio in the processed cheeses is only 1:3 because of the administration of processing salt. To improve this ratio, the HDRI has developed a new processed cheese without peptisation, with a 3:1 Ca:P ratio [5].

As a consequence of the above described preliminary events we have started an over-all research work in 2000, which has resulted in the development of series of probiotic and Ca^{2+} enriched foods.

Experimental

Materials and methods

To detect and prove the probiotic characters of milk-acid strains isolated by HDRI, in vitro and in vivo methods were used.

We have determined during in vitro investigations the optimal proliferation temperature of the strains, their cholesterol decomposition, their resistance against acid and bile acid as well as their exopolisaccharide (EPS)-production.

During in vivo investigations till 4 weeks 15 persons consumed traditional kefir, and we have administrated probiotic kefir (SynBiofr) for 57 persons, in 0.5 L day^{-1} dose. We have determined their lipid profile as well as their strain composition before the starting of consumption and at the end of the 2nd and 4th week respectively. Differential scanning calorime-

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ter (Setaram Micro-DSC-II, France) was used in isotherm mode for the side by side detection of probiotic and other lactic acid strains. We have measured the isotherm heat production of proliferation. The heat flow curve was decomposed into Gaussian curves, and the microbe ratio was determined from the area under the decomposed compounds.

It was chosen basic raw foodstuffs for the development of Ca-enriching material that way, that itself contains huge amount Ca and contains those adjuvant which promote the absorption of Ca and its building into the bones.

Using the developed Ca-enriching additive-food and administrating Ca-enriched fermented milk, we have proved with human clinical investigations the absorption of Ca and its building into the bones. The measure of absorption was determined from the Ca-content of urine; the building into the bones was monitored with biomarkers [Parathormon (PTH), osteocalcine (BGP), piridinolin cross bindings in the serum (PLC_1-D) and in the urine (PLC_2-D), urine creatinin (UC) and PLC_2-D/UC ratio].

During the development and production of probiotic and Ca-enriched foodstuffs we have determined those other functional characteristics which increase the consumption value of the new products over the originally planned characteristics too.

We have applied a so called 'focal-group' during the development the new products to estimate how will accept the market them.

Results and discussion

We have determined from the in vitro measurements of slime-producing strains isolated by HDRI that

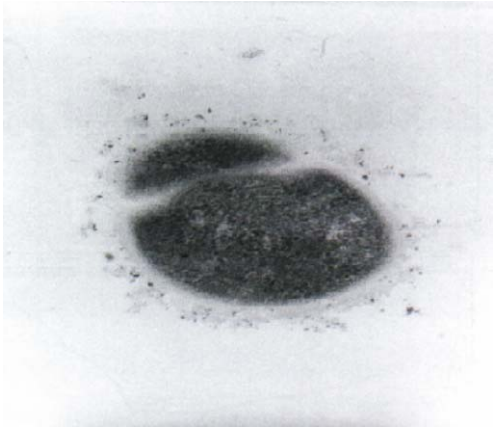

Fig. 1 An electronmicroscopic photo of an EPS-producing microbe

- the proliferation temperature-optimum of strains corresponds to the human body temperature: 36–38°C,
- they digest 20–30% more cholesterol during fermentation than other milk acid bacteria,
- their 80% survive at low pH (~2.3) and at the actual concentration of bile acid of the digesting system, furthermore
- this strain early named as slime, is a viscosity increasing material and it is an EPS.

In Fig. 1 can be seen an electron microscopic picture of a proliferating microbe, in the market named as Prebiolact-2. In the picture can be seen the exopolisaccharide cloud in the surrounding of the microbe. This EPS will result later the increase of the viscosity of the product.

The results of in vivo experiments proving the probiotic characteristics of isolated microbes are summarized in two tables. The lipid-profiles of blood samples of investigated patients can be found in Table 1, while the micro flora of their faces are pre-

Table 1 Change of lipid-profile of blood samples of persons consuming traditional Russian-type and probiotic kefir developed by HDRI during a four-week clinical examination

Examination group	parameters	units	Blood plasma's examined: average values			level of significance	
			0	2	4	2	4
			time elapsed/week				
Consumers of traditional kefir (control) *n*=15	total cholesterol	mM L^{-1}	7.01	7.0	6.83	NS	
		change %	–	–0.1	–2.6		
	triglyceride	mM L^{-1}	2.07	1.90	2.08		
		change %	–	–8.2	+0.5		
SynBiofir consumers (experimental) *n*=57	total cholesterol	mM L^{-1}	7.16	6.81	6.76	$p<0.001$	
		change %	–	–4.9	–5.6		
	triglyceride	mM L^{-1}	2.62	2.34	2.19	$p<0.01$	$p<0.001$
		change %	–	–10.7	–16.4		

Table 2 Change of essential microflora of fecal samples of persons consuming traditional Russian-type and probiotic kefir developed by HDRI during a four-week clinical examination

Exam. gr.	Number of persons	Microbe group	Unit	Microbe count of fecal samples		
				0	2	4
Consumers of traditional kefir (control) *n*=15	15	Total plate count (aerob+anaerob)	10^6 g^{-1}	10585	7862	45125
			index	1.0	0.7	4.3
		Probiotic total	10^6 g^{-1}	946	608	1208
			index	1.0	0.6	1.3
			ratio %	8.9	7.7	2.7
		– Streptococcus	10^6 g^{-1}	220	374	285
			index	1.0	1.7	1.3
		– Lactobacillus	10^6 g^{-1}	226	37	164
			index	1.0	0.2	0.7
		– Bifidobacterium	10^6 g^{-1}	500	197	759
			index	1.0	0.4	1.5
SynBiofir consumers (experimental) *n*=60	60	Total plate count (Aerob+anaerob)	10^6 g^{-1}	4360	38278	29785
			index	1.0	8.8	6.8
		Probiotic total	10^6 g^{-1}	554	4397	21440
			index	1.0	7.9	38.7
			ratio %	12.7	11.5	72.0
		– Streptococcus	10^6 g^{-1}	134	593	930
			index	1.0	4.4	6.9
		– Lactobacillus	10^6 g^{-1}	78	81	96
			index	1.0	1.0	1.2
		– Bifidobacterium	10^6 g^{-1}	342	3723	20414
			index	1.0	10.9	59.7

sented in Table 2. These data present quite well that the consumption of traditional kefir does not have practically any affect on the blood lipid-profile or on the micro flora of the faces. In contrast, both parameters changed significantly consuming product made with Prebiolact-2 culture, containing isolated strains. The total cholesterol level of blood decreased (by 5.6%) and significantly decreased the triglyceride level too (by 16.4%). In harmony with it, the probiotic germ ratio in the faces significantly increased (from 12.7% to 72.0).

The mixture of probiotic and non-probiotic microbes could be detected simultaneously with the aid of isotherm calorimetry. The deconvoluted DSC scan of testing a Prebiolact-2 and butter culture mixture can be seen in Fig. 2, while the results are summarized in Table 3. We could determine from the two characteristic Gaussian curves of Fig. 2 that during the fermentation in 75% Prebiolact-2, in 25% microbes of butter culture have been infested.

We have used acerbic curd whey during the development of Ca-enriching additive food, which generally is taken as a 'side-product' in the dairy technology. This product is unique in Europe; it is the result

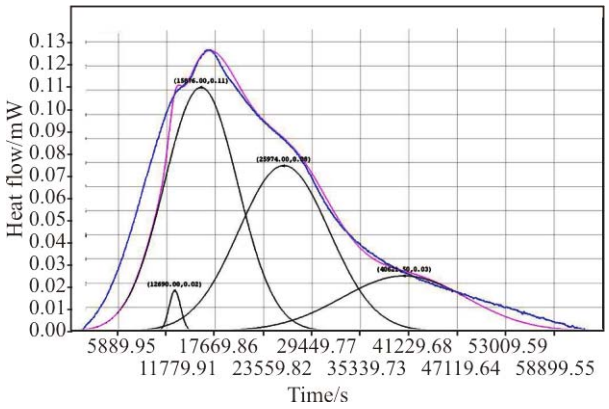

Fig. 2 A typical deconvoluted DSC-curve of the mixture of pure cultures of probiotic and non-probiotic lactic acid bacteria

of a typical Carpathian basin technology to produce curd with pickling fermentation. The Ca will be split off under the effect of acidification and after the pouring out slowly the Ca content of whey will be greater than that of base milk. The main components of curd whey powder with a marketing name Kalcima® QC that is developed by us [6] can be seen in Table 4. The

Table 3 DSC-data and microbe counts

Name of sample	S	H	H/%	M	C·10^8	H°	H°/C·10^{-8}	Ratio of culture/%
	12096	63	2.8					
Prebiolact	16686	1495	66.7	0.564	5.4	2651	558	100
	30767	681	30.4					
Butter culture	7344	344	10.7					
	23423	2846	89.3	0.559	4.2	5091	1084	100
	12690	33	1.2					
Mixed culture	15876	1246	44.4	0.576	5.9	2163	Prebiolact	75
	25974	1046	37.3			1816	Butter culture	25
	40622	480	17.1					

S: Time of maximum values of Gaussian-curve, s; H: The area below Gaussian-curve (heat production), mJ; H%: The ratio of area below Gaussian-curve in %; M: Mass of sample examined by DSC, g; C: Total plate count of the product fermented to pH 4.5; H°=H/M: The area normalised to 1 g; H°/C: Heat amount equivalent to development of one microbe

Table 4 The most important chemical characteristics of KALCIMA®QC skimmed quarg whey powder

Chemical characteristics		
Name	units	average
Dry matter	%	≥96.0
Moisture	%	≤4.0
Milk sugar (lactitol, lactulose)7	%	28.0±5.0
protein	%	3.8±0.8
fat	%	≤1.0
calcium (Ca)	%	11.0±1.0
phosphor (P)	%	0.53±0.03
Ca:P ratio	rel.	20:1
magnesium (Mg)	%	0.18
potassium (K)	%	1.40
sodium (Na)	%	0.51
manganese (Mn)	mg(100 g)$^{-1}$	0.38
copper (Cu)	mg(100 g)$^{-1}$	0.15
cinc (Zn)	mg(100 g)$^{-1}$	2.1
From Ca-salts:	%	
-organic bond (malate, lactate, citrate)	%	92
-inorganic bond (phosphate)		8
Heavy metals: As	mg kg^{-1}	<0.5
Hg	mg kg^{-1}	<0.01
Pb	mg kg^{-1}	<1.0
Cd	mg kg^{-1}	<0.5
Acidity	pH	~ 5.5

data of Table 4 represent quite well that this additive food with its 11% Ca content is a good material to enrich the foods in Ca, it could be used because of its 20:1 Ca:P ratio to equalize or to make positive the negative Ca:P ratio of foods. Its other constituents (milk sugar, oligosaccharides, Mg) as adjuvant can help to built in the Ca into the bones.

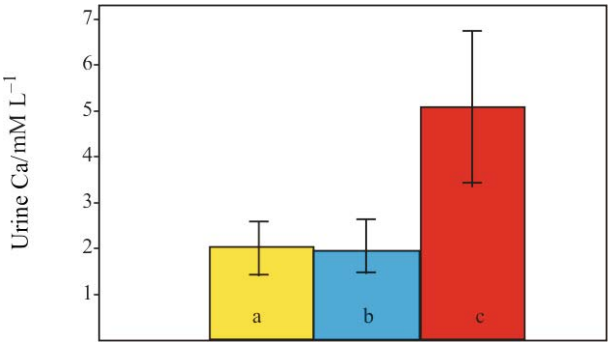

Fig. 3 Ca-absorption from a – a normal diet and from fermented milk enriched (1100 mg person^{-1} surplus Ca) with b – inorganic Ca and c – Kalcima®QC (18–18 persons)

The absorption of Ca from fermented milk enriched with Kalcima® QC can be seen in Fig. 3, and the change of biomarkers indicating its built in into the bones can be found in Table 5. Figure 3 demonstrates quite well that the Ca – in contrast with inorganic Ca salt – is sucked up from the food enriched with Kalcima® QC, and Table 5 shows that the change of biomarkers is the indicator of the built in ability of sucked up Ca into the bones (greater is the decrease, bigger is the probablity of building in).

Developed products, their evaluation and conclusions

Probiotic kefir

The basis of the developed procedure is that the technology assures the propagation of the germs both of the mesofil kefir-culture and of the thermofil probiotic culture during the production [7]. This way the end product has a taste effect characteristic for the kefir, but it has greater viscosity and water binding

Table 5 Change of blood and urine biomarkers indicating bone turnover of osteoporotic but in all other respects healthy persons above 50 in groups medically treated (control) and consuming Kalcima®QC from fermented dairy product (experimental) during six weeks

Biomarker examined	Degree of change after six weeks compared to the initial value, %		
	medically treated	400 mg person^{-1} day^{-1}	800 mg person^{-1} day^{-1}
		consuming Kalcima®QC in fermented dairy product	
	control (n=11)	experimental (n=12)	
Parathormon (PTH)	–5.8	–5.6	–16.9
Oszteokalcin (BGP)	–6.3	–10.5	–18.2
Piridinolin crossbridges in serum (PLC1-D)	–13.4	–63.9	–58.6
Piridinolin crossbridges in urine (PLC2-D)	–0.1	–24.2	–17.3
Urine creatinin (UC)	–5.3	–20.0	–19.4
PLC2-D/UC	6.1	–6.8	–11.1

capability. The results of preliminary marketing survey have proved that the greater viscosity characteristic of the product is positively evaluated by the consumers. This product with a name Synbiofír is in the market since more years; its consumption rate is steady because of its wide penetration in the trade.

Probiotic sour cream

The basis of the developed procedure is that the cream is fermented together with the EPS producing probiotic and butter culture, after it, it will undergo to such a mechanical influence that will break up the strong protein–protein bonds, after that they will be restored in weaker form but in greater number. The gel, developed this way has the same firmness as it was at the beginning of the procedure, but it could be more easy whipped, and it will not precipitate in hot food [8]. The preliminary tests of the consumption survey have proved that the consumers judged the probiotic cooking resistant sour cream from all points of view better than that of currently circulating one: it has the same firmness, it could be better whipped, it is precipitated fewer manners in hot food and it better whitens. Its probiotic characteristic was especially accepted first of all in the production and consumption of cold dishes (e.g. salads).

This product was in the market in Hungary only during the years of 2002–2005, but its marketing was very bad, no one of its advantage characteristic was advertised. This way it did not result in market advantage, therefore its production was finished. Our recent aim is to introduce this product that way that the consumers should be properly informed.

Probiotic butter creams with living flora

The basis of the developed procedure is that we can improve the spread ability of traditional butter creams without adding butter. We use only cream stock, we exploit the EPS production of probiotic culture, and we set the parameters of homogenization and properly choose the characteristic of food additives. This way the sample could be coagulated in jar without post heat-treatment [9]. A further nutritional physiological advantage beside the probiotic characteristic is the only use of cream stock of great dispersity degree that increases further the digestibility of the product. This could be seen in Fig. 4, where the digestibility of six fat products are demonstrated referring to the lard (100%) digestibility. It could be seen from the figure that the fat from butter cream of living flora has better digestibility (the absorption is

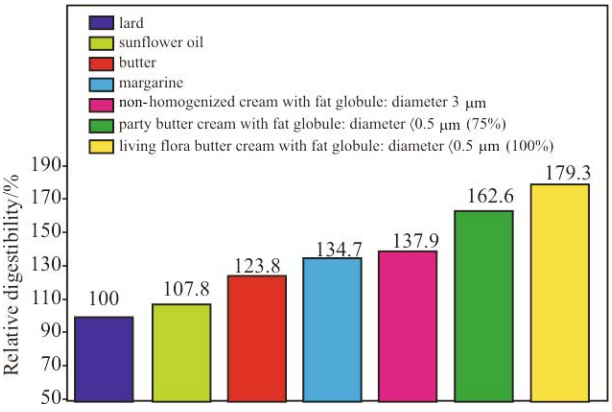

Fig. 4 Relative digestibility (rate of absorption) of fat originating from live flora probiotic butter cream (fat globule diameter 100%<0.5 μm) and other six foods (lard, sunflower oil, butter, margarine, non-homogenized cream with fat globule diameter 3 μm and Party butter cream with fat globule diameter 75%<0.5 μm) in third h after consumption (lard=100%)

quicker) than in case of traditional butter cream. It comes from the fact that the average diameter of fat balls in the whole fat phase is smaller than 0.5 μm.

The results of preliminary trade survey prove clearly that the consumers like much better the probiotic butter creams than the traditional one even though its consumer price would be more expensive than that of traditional one. Despite of the fact that the production price of probiotic butter creams of living flora is about 15–20% smaller than that of others until now we could not realized the product and technology introduced in 2001. To analyze the reason of it we can say that the recently traded butter cream either in quantity or in profit producing capability fits to the demands of the persons involved in the market, therefore there is a resistance against of any kind of change.

To introduce into the market our new product we have to change our strategy: we have to look for producers who are not present recently in the butter cream market, and after it we should circulate our product. This marketing strategy is in process.

Ca-enriched meat products

The basis of the developed procedure is that to accommodate to the preparation of meat product (made by cutter or tumbler, prick off, breading) we have to administrate into the stock of meat product such amount food additive at the proper technological point that the Ca:P ratio of the end product should be at least 1:1 [10]. The results of the preliminary trade survey prove that the consumers can not distinguish between Ca enriched and traditional meat products with their organ of sense, and in case of equal prices they would buy Ca enriched products. The Ca enrichment could be proposed in case of meat products made from poultry meat (e.g.: poultry wienerwurst, -ham, -cold cuts) because their Ca:P ratio is the worst nowadays (1:30–1:40). Recently Ca enrichment is used in case of quick frozen turkey chest products.

Ca-enriched baking industrial products

The basis of the developed procedure is that we mix food additives to the flour to reach the wanted Ca:P=1:1 ratio, after it we make the production with the technology of the traditional product. We have observed that this way other functional characteristics of Ca-enriched baking industrial products (e.g.: roll, loaf) improved: we have got greater volume in case of equal masses and according with it the dough was more slack and the surface was more uniform backed. The results of preliminary trade survey show that the consumers realize the better quality, but they would buy the Ca-enriched product instead of traditional one only in case of unchanged price. With respect to the increased cost in case of the production of Ca-enriched baking industrial products (more constituents, greater price) we can not propose the bulk-production, but it could be imagined in case of new products for special nutritional demands.

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