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INDICATION OF PROLIFERATION OF LACTIC ACID BACTERIA IN MIXED CULTURES
Estimation of their ratios by isoperibolic batch calorimetry

*Bernadett Litz and B. Schäffer**

Hungarian Dairy Research Institute, Tüzér str. 15, 7623 Pécs, Hungary

The supply and consumption of probiotic foods, and particularly probiotic dairy products, has grown steadily in recent years. In the production of dairy products of this type other microbes must also be used in addition to the microbes which provide the probiotic effect and which generally have a proliferation optimum at 37°C. The probiotic microbes have a neutral taste in dairy products consequently the taste of fermented dairy products is supplied by other microbes. These microbes are likewise lactic acid bacteria, and their proliferation optima are either below (mesophilic) or above (thermophilic) that of the probiotic microbes. It is imperative to have an indication of whether the probiotic bacteria have multiplied at the fermentation temperature used during the technology, since they provide the beneficial physiological effect of the product. Isothermic calorimetry appeared a suitable method for the indication of this process, because the amount of heat released during lactic acid bacterial proliferation differs from the probiotic one. In order to analyze the heat flow curves a deconvolutional program was developed which decomposed them into Gaussian curves, because the proliferation of individual microbes follows a lognormal distribution. The Gaussian curve characteristic for the culture was determined, and from the area under the curve the heat liberated during the creation of one microbe was calculated.

Keywords: *deconvolution, isoperibolic batch calorimetry, lactic acid bacteria, probiotic*

Introduction

In the field of sour dairy products the consumption of probiotic products is steadily increasing throughout the world. Health protective effect of dairy products containing probiotic microbes in an appropriate amount has been clinically proved. The probiotic strains tolerate acid and bile salt, proliferate in colon and suppress the proliferation of harmful bacteria [1–3]. As a consequence of these properties they have health protective functions such as restoration of useful intestinal flora after treatment with antibiotics, decrease of blood pressure, decrease of cholesterol level, prevention of colon cancer etc. [1–3].

Dairy products fermented exclusively by probiotic microbes are of neutral taste. However, the consumers require probiotic dairy products with the familiar characteristic flavour that evoke a same impression and taste as the traditional products, therefore, it is inevitable for dairy industry to produce dairy products which are both in name and function probiotic e.g. yoghurt, kefir, sour cream, butter cream or cheese spread products [4].

That is why the probiotic dairy products contain at least two different cultures, but their growing optima generally do not coincide. The growing optimum of probiotic cultures is usually the body temperature (37°C), but the culture determining the character of the product is either mesophilic (20–24°C), or thermophilic (42–45°C) of nature. There are three possibilities in the production technology: co-fermentation, fermentation separately followed by mixing and post inoculation by probiotic microbes. However, there is the question in all the three cases whether are in the final product probiotic microbes, and if yes, in what ratio. Considering that the microbes determining the character of the product and microbes of probiotic effect are morphologically often the same (e.g. of coccus shape), their indication in mixed culture is hardly difficult (e.g. possible by gene examination).

Beside the well established microbiological and DNA sequences methods, differential scanning and isotherm (isoperibolic) calorimetry represents a useful approach in this field. It allows to demonstrate the thermal consequences of any conformational changes in the structure of medium including the proliferation processes too. This technique has already proved to be widely accepted in the research of thermodynamic characteristics of various biological systems such as: muscle proteins in different intermediate states of ATP hydrolysis cycle [5–16], human cartilage and vertebrate discs [17–19], abnormalities of human leg skeletal muscles [20], dog trachea [21] as well as in physiological and microbiological research [22, 23].

* Author for correspondence: mtkipecs@axelero.hu

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Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

We have experience with this method in dairy product development too [24–27].

During recent experiments we wanted to clarify whether the heat produced by microbes can be detected and their ratio could be determined from the measured heat flow. However, by differential scanning calorimetry, first of all, the inactivation of microbes [28] and denaturation of bacterium cell [29, 30] are examined. The proliferation of microbes is an exothermic process in isotherm conditions. The time belonging to the maximum point of heat flow curve corresponds to the time belonging to the inflexion point of the bacterium proliferation curve [31]. The basic procedure for separation of different bacterium cultures on the basis of proliferation heat flow curve was the determination of the characteristic proliferation curve and the heat produced during the proliferation.

Materials and methods

Dairy samples

In experiments we examined the functional product properties and the most important cultures providing probiotic effect which were the following:

Cultures providing functional product properties

- Butter culture (sour cream, butter cream, cheese spread)
- Kefir culture (natural and flavoured kefirs)

Probiotic cultures

- Prebiolact-2 (probiotic culture isolated by us ourselves)
- Bifidobacterium bifidum (available in commercial turnover)

Isothermal calorimetry

During experiments approximately 450 mg sterile fat-free milk and about 50 mg culture were measured into a mixing batch vessel (SETARAM). The samples were placed into a SETARAM Micro DSC-II calorimeter, after it we waited till the thermal equilibrium was reached at the characteristic temperature of the culture, i.e. at 30°C. As a reference we used distilled water prepared in the same way in order to prevent the microbe proliferation in the reference cell. Reaching the thermal equilibrium we have mixed the two components and the heat flow curves of microbe proliferation were recorded in isotherm condition at 30°C.

Data handling of heat flow curves

In order to analyze the heat flow curves a deconvolutional program was developed which decomposed the original curves into Gaussian functions [32]. The deconvolution into Gaussian curves was chosen because the microbes in the culture proliferate according to separate S-curves and their first differential quotient is a Gaussian curve.

Microbiological test

For determining the total plate count thousand-fold quantities were used: 450 g fat-free sterile milk was inoculated by 50 g culture at 30°C, then it was fermented at 30°C till pH=4.7 was reached (at Bifidobacterium bifidum culture for 12 h), the fermented milk was cooled to 4°C, then within 24 h the total plate count was determined according to the recommended international standard methods.

Results and discussion

As far as we know we have made the first attempt on dairy product to separate the proliferation of probiotic bacteria from the others by determining the impact of the ratio of exopolysaccharide-(EPS)-producing microbes identifiable by isoperibolic calorimetry on the functional properties of heat-resistant sour cream. We have developed for the separation of a deconvolution program [32] which has the next main steps:

- Correction of the baseline,
- Searching for Gaussian functions to have a rough fitting,
- Determination of parameters of fitted Gaussian functions and
- Parameter correction to have the best fitting.

The program itself using the initial and final point of proliferation, chosen by us, makes the baseline correction. Gaussian functions were fitted in the first step to the local maximum places. The estimation of parameters is given by numeric method, parameter correction is made by multiple running of fitting which is going on till the difference between the experimental and simulated total heat flow curve will be the minimum. The program displays graphically the original and the fitted heat flow curve, the Gaussian curves, and calculates the places of maxima, their heat flow values, halfwidth of Gaussian curves, the area under the Gaussian curves and their area ratios referring to the total proliferation heat flow.

Figure 1 shows the isotherm heat flow curve of a butter culture proliferation as an example.

It can be seen from the figure that the butter culture proliferates well at 30°C and the microbes being

Table 1 Data of DSC curves and microba counts

Sample	*t*/s	*H*/mJ	*H*/%	*M*/g	$C \cdot 10^6$/Cfu
Butter culture	7 344	344	10.7	0.559	4 200
	23 423	2 846	89.3		
Kefir culture	28 400	2 220	96.3	0.562	3 500
	32 600	85	3.7		
Prebiolact-2 culture	12 096	63	2.8	0.564	5 400
	16 686	1 495	66.8		
	30 767	681	30.4		
Bifidobacterium bifidum	45 400	2	100.0	0.602	3

t is the time belonging to the matched Gaussian curves in s; *H* in mJ is the area of Gaussian curves that is the heat production; *H*% stands for the percentile ratio of the Gaussian curves in the total heat flow; *M* is the total mass of sample (skim milk plus probiotic culture) in g; *C* stands for the total plate count measured after fermentation till pH=4.7 (or for 12 h) in Cfu g^{-1} unit

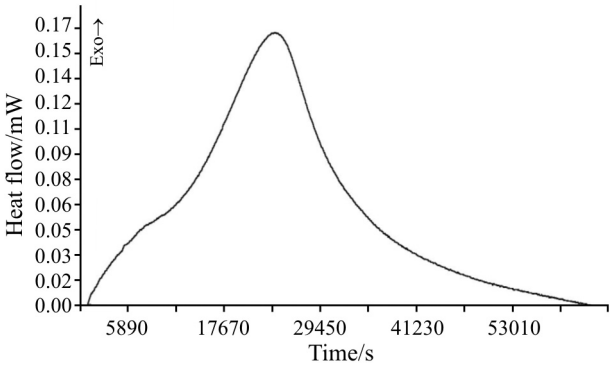

Fig. 1 DSC curves of butter culture

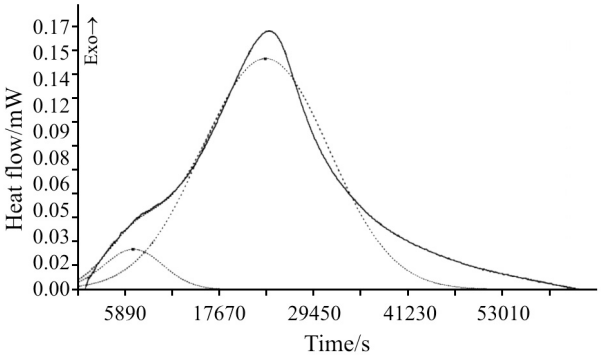

Fig. 2 Deconvoluted DSC curve of butter culture

in the butter culture attain their proliferation maximum at this temperature within 6.5 h. It is also shown by the curve that the butter culture consists of several microbe strains as far as the curve is not a symmetrical Gaussian curve, but obviously is a resultant of some Gaussian curves.

Figure 2 shows the deconvoluted heat flow curve. It is obvious that the heat flow curve could be decomposed into two Gaussian curves, the maxima of which are at the 2nd and 6.5th hours of proliferation.

The microbe count of different cultures at pH=4.7 and after fermentation of 12 h were the following:

- Butter culture: $4.2 \cdot 10^8$
- Kefir culture: $3.5 \cdot 10^8$
- Prebiolact-2 culture: $5.4 \cdot 10^8$
- Bifidobacterium bifidum culture: $2.5 \cdot 10^6$

Isotherm heat flow and microbe count data necessary to draw conclusions are summarized in Table 1 where we have listed data for different cultures which could be taken into account during the production of probiotic dairy products. Only the most reasonable Gaussian curves are presented in each case. The basis of selection was the fact that in case of mixed cultures providing both the most characteristic product traits and the probiotic property these Gaussian curves should be possibly the farest from each other and possibly having the biggest contribution to the heat production.

Table 2 Calculated data of heat production

Sample	H_0/mJ g^{-1}	$H_0/C \cdot 10^{-8}$/mJ Cfu
Butter culture	5091	1212
Kefir culture	3950	1129
Prebiolact-2 culture	2651	491
Bifidobacterium bifidum	3.3	1100

From the data of Gaussian curves presented in Table 1 following data have been calculated:

- Heat production during proliferation referring to 1 g sample ($H°$) in mJ g^{-1} unit and
- Heat production corresponding to a single microbe creation (or microbe colony) ($H°$/C), in mJ Cfu^{-1} unit.
- The results are shown in Table 2. Its data could be well used at experiments where the ratio of functional and probiotic microbes of different probiotic effect dairy products should be determined.

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