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The influence of fat and monoacylglycerols on growth of spore-forming bacteria in processed cheese

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ABSTRACT

Highly undesirable microbial contaminants of processed cheese are endospore-forming bacteria of the genera *Bacillus* and *Clostridium*. Survival of *Bacillus subtilis, B. cereus, Clostridium butyricum* and *C. sporogenes* was examined in model processed cheese samples supplemented with monoacylglycerols. In processed cheese samples, monoacylglycerols of undecanoic, undecenoic, lauric and adamantane-1-carboxylic acid at concentration of 0.15% w/w prevented the growth and multiplication of both *Bacillus* species throughout the storage period. The two species of *Clostridium* were less affected by monoacylglycerols in processed cheese samples and only partial inhibition was observed. The effect of milk fat content on microbial survival in processed cheese was also evaluated. The growth of *Bacillus* sp. was affected by the fat level of processed cheese while population levels of *Clostridium* sp. did not differ in processed cheese samples with 30, 40 and 50% fat in dry matter.

1. Introduction

Processed cheeses are cheese-based foods produced by comminuting, melting and emulsifying natural cheeses and optional ingredients into a smooth homogenous molten blend using heat, mechanical shear and emulsifying salts. Cheese processing normally involves the heat treatment of ingredients at a temperature of 80–100 °C which is held for 5–15 min. During this process the majority of vegetative forms of microorganisms are inactivated. Nevertheless, the temperatures used, are not sufficient for killing bacterial endospores, although the spore-forming microorganisms are often weakened (Glass et al., 1998). Hence, processed cheese products may contain viable spores which originate in the natural cheese or other ingredients. From a microbiological point of view, the most significant contamination of processed cheese is caused by gram-positive spore-forming bacteria of the genus *Bacillus* and *Clostridium* (Glass and Doyle, 2005; Lycken and Borch, 2006). Microbial quality of processed cheese products depends on the quality of ingredients, as well as on other factors such as pH, moisture, fat, salts or the presence of additives (Glass and Doyle, 2005; Tersteeg et al., 1995). The growth of undesirable microbial flora in food products can be limited or prevented also through application of antimicrobial compounds (Davidson et al., 2005).

Research on antimicrobials, especially naturally occurring compounds, has increased dramatically in the past 10 to 15 years. The primary incentive for searching for effective antimicrobials among naturally occurring compounds is to expand the spectrum of antimicrobial activity over that of the traditional, regulatory-approved substances. Interest in natural antimicrobials is also driven by the fact that there is a current worldwide drive for a healthier lifestyle, which has led to a rising demand for fresh foods, free from "chemical additives".

One group of antimicrobial compounds found in nature and considered to have little or no toxicity is the fatty acids and their corresponding esters (Fagan et al., 2004; Davidson et al., 2005). These compounds include monoacylglycerols that are widely used for their emulsifying abilities in the food processing industry (Gunstone, 2004; Whitehurst 2004). As naturally occurring compounds, mono- and diacylglycerols of fatty acids hold a GRAS (generally recognised as safe) status in the United States and within the EU they are generally permitted for use in food products (Davidson et al., 2005; Whitehurst, 2004). Monoacylglycerols of various fatty acids have showed promising activity against diverse microorganisms including gram-positive and gram-negative bacteria (Altieri et al., 2009a; Buňková et al., 2011; Kabara et al., 1972; Preuss et al., 2005; Růžička et al., 2003), spore-forming bacteria (Mansour et al., 1999; Chaibi et al., 1998), yeasts (Bergsson et al., 2001; Růžička et al., 2003), filamentous fungi (Altieri et al., 2009b; Buňková et al., 2010; Růžička et al., 2003) and also enveloped viruses (Hilmarsson et al., 2005). Antibacterial activity of monoacylglycerols has been also proven in the environment of foods and beverages (Doležálková et al., 2012; Mansour et al., 1999; Nair et al., 2004).

In a previous study Buňková et al., 2011 compared the inhibitory effects of seven monoacylglycerols (MAG) containing fatty acids with a

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medium chain length on ten strains of food-borne pathogens or spoilage gram-positive and gram-negative bacteria. Within the monoacylglycerols tested, MAGC11:0, MAG C11:1 and MAGC12:0 showed inhibitory effects on the growth of gram-positive bacteria at very low concentration (25 μg/ml). Moreover, application of monoacylglycerols in foods seems to be an attractive choice since they could serve as antimicrobials and as an integral part of foodstuffs they could even improve functional properties of dairy or bakery products (Buňka et al., 2007; Ravi et al., 2000). These facts and previous results with gram-positive bacteria in laboratory media led us to study the effect of MAGs on gram-positive bacteria inoculated in processed cheese. Except for monoacylglycerols of fatty acids, monoacylglycerol containing adamantane moiety was chosen for studying its antimicrobial activity in processed cheese.

Survival of *Clostridium* sp. and *Bacillus* sp. vegetative cells and spores was examined in model processed cheese samples. In one series processed cheese samples differing in fat content were produced in order to evaluate the influence of fat on microbial growth. To evaluate antimicrobial activity of monoacylglycerols, processed cheese samples were treated with monoacylglycerols selected according to their antimicrobial activity *in vitro*.

2. Material and methods

2.1. Bacterial strains

Bacillus cereus CCM 2010 and *B. subtilis* subsp. *spizizenii* CCM 4062 were obtained from the Czech Collection of Microorganisms (CCM, www.sci.muni.cz/ccm/). *Clostridium butyricum* CAPM 6342 and *C. sporogenes* CAPM 6329 were obtained from Collection of animal pathogenic microorganisms (CAPM, Veterinary Research Institute in Brno, Czech Republic).

Suspensions of *Clostridium* sp. and *Bacillus* sp. were prepared by inoculating 10 ml of nutrient broth (HiMedia, India) by 25 μl of one-day inoculum ($\approx$4.6–8.2 · 10^6 CFU/ml) of the corresponding strain. Bacteria were subsequently grown at 37 °C for 48 h.

To prepare spore suspensions cultures of *Clostridium* sp. and *Bacillus* sp. were incubated aerobically for *Bacillus* species and anaerobically for *Clostridium* species at the appropriate temperature for each microorganism until sporulation. When free spores were observed microscopically, they were harvested from the plate surface by washing it four times with sterile distilled water, centrifuging the washings at 10,000 rpm for 15 min at 4 °C, resuspending the pelleted spores in 10 ml of sterile distilled water and then heating the suspension to 80 °C for 10 min.

2.2. Monoacylglycerols

Monoacylglycerols were prepared by the direct addition of particular acid to glycidol ((oxiran-2-yl)methanol) by nucleophilic opening of the epoxide ring of glycidol according to the procedure published by Janiš et al. (2006). The reaction was performed under fixed conditions of pressure and temperature in an open glass reactor equipped with magnetic stirrer and temperature-stabilised jacket capable of maintaining the reaction temperature within ±0.5 °C limit. Reaction temperature for the preparation of monoacylglycerols of undecanoic, undecenoic and lauric acid was 90 °C. Monoacylglycerol of adamantane-1carboxylic acid was prepared according to Doležálková et al. (2012). Fatty acids, adamantane-1-carboxylic acid and glycidol were purchased by Sigma-Aldrich (St. Louis, USA). Crude reaction products were purified by threefold filtration and consequential recrystallization from ethanol. Purified monoacylglycerols were analyzed by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) to evaluate the content of unreacted components and purity of the product. These analyses did not prove either significant formation of side products or reactant residues. Monoacylglycerols selected for this experiment were: (i) monoacylglycerol of undecanoic acid (MAG C11:0); (ii) monoacylglycerol of undecenoic acid (MAG C11:1); (iii) monoacylglycerol of lauric acid (MAG C12:0) and (iv) monoacylglycerol of adamantane-1-carboxylic acid (MAG ACA).

Stock solutions were prepared by dissolving 0.2 g of MAG in 10 ml of 96% ethanol and sterilizing by filtration through 0.22 mm filters (Millipore, UK) before storing at 4 °C.

2.3. Processed cheese production

Production of processed cheese samples consisted of several steps. At first formulation and selection of the different types and levels of ingredients were carried out. In one series of experiment processed cheese samples differing in fat content were produced in order to evaluate the influence of fat on microbial survival. Levels of ingredients are specified in Table 1. Then the ingredients were blended with water with subsequent processing by heating and shearing. Processing was carried out using Vorwerk Thermomix TM 31-1 (Vorwerk & Co. Thermomix; GmbH, Wuppertal, Germany) at temperature 85–90 °C held for 1 min. In the next step, samples were homogenized, packaged and cooled. Before storage, the samples were inoculated with 5 ml suspensions of vegetative cells or spores of particular microorganism (ca 2.5–4.8 . 10^5 CFU/ml). Immediately after melting and inoculation with the microorganism, the melt was packed into sterile cylindrical polypropylene cups (52 mm in diameter; 50 mm high), closed with sterile aluminium foil and cooled to 6 °C, within 2 h. The cooled samples were stored at 6 ± 2 °C. To determine the effect of MAGs on spore-forming bacteria in cheese samples, processed cheese samples of 50% fat in dry matter were supplemented with monoacylglycerols at concentrations of 0.01%, 0.05% and 0.15% (w/w). These concentrations were selected considering two aspects, antimicrobial activity of MAGs *in vitro* and sensory properties of processed cheese samples. Control cheese without MAG addition was prepared separately for each experiment (e.g. cheese samples containing MAG C11:0). Samples were sensorically evaluated (only appearance and consistency) by means of 3 expert assessors, employees of Department of Food Technology trained according to ISO 8586–2, 2008.

2.4. Enumeration of microorganisms and evaluation of results

During storage, processed cheese samples were removed from incubation at the selected time intervals and microbiologically analyzed. In each case, 5 g of each sample was aseptically removed, diluted 1:9 in saline solution and homogenized for 2 min with a stomacher. Serial dilutions in saline were made and 1000 μl of an appropriate dilution was poured into Petri dishes along with cultivation medium tempered to 50 °C. Plates were incubated for 48 h at 37 °C. After incubation, aerobic and anaerobic plate counts were determined and the number of colony forming units per one gram of processed cheese sample (CFU/g) was calculated. Data were expressed as mean values of log CFU/g of three replicate experiments (three lots of cheese and three independetly prepared cultures). Aerobic population of *Bacillus* sp. levels were determined using Plate Count Agar (PCA, HiMedia) with incubation at 37 °C for 48 h. Anaerobic plate counts for *Clostridium* sp. were performed

Table 1
Composition of ingredients for processed cheese production.

fat in dry matter (%)	30%	40%	50%
	mass (g)		
edam cheese*	800	650	550
butter**	15	90	170
DIDI (Na_2HPO_4. $2H_2O$)	21	21	21
KPS ($Na_2H_2P_2O_7$)	6	6	6
PYRO 52 ($Na_4P_2O_7$)	3	3	3
water	255 ml	300 ml	355 ml

* Edam cheese — Dutch type cheese, approximately 50% (w/w) dry matter, and 30% (w/w) fat in dry matter; age 4 weeks.
** Butter — approximately 84% (w/w) dry matter and 82% (w/w) fat.