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ANTIMICROBIAL ACTIVITY OF NON-TRADITIONAL MONOACYLGLYCEROLS

ANTIMIKROBNÍ ÚČINKY NETRADIČNÍCH MONOACYLGLYCEROLŮ

Doctoral Thesis

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ABSTRACT

Limiting or preventing the growth of undesirable microbial flora in food products is one of the main goals of food microbiology. A number of preservation methods are designed to extend the shelf-life of foods by reducing the microbial growth and new antimicrobials are still examined. Monoacylglycerols are naturally occurring compounds with inhibitory activity against various microorganisms and they seem to be an attractive choice since they could serve as antimicrobials and they could even improve functional properties of foods.

The presented thesis focuses on antimicrobial activity of monoacylglycerols against food-borne microorganisms. Seven monoacylglycerols differing in the structure of acid bound have been prepared for testing their potential antimicrobial effect *in vitro*. These include monoacylglycerols of fatty acids with even or odd number of carbon atoms, monoacylglycerols of unsaturated fatty acids and monoacylglycerols of non-traditional acids. Results of antimicrobial assays showed a dependence of antimicrobial activity on the nature of acid esterified to glycerol backbone. Food-borne pathogenic and spoilage microorganisms tested *in vitro* were sensitive especially to monoacylglycerols containing fatty acids with 10, 11 and 12 carbon atoms. Except for a decrease in population density caused by monoacylgycerols, lag-time extension and decline in specific growth rate occurred. Based on *in vitro* results, monoacylglycerols with the widest antimicrobial spectrum were evaluated for their action in processed cheese samples. Highly undesirable microbial contaminants of processed cheese are rod-shaped endospore-forming bacteria of the genera *Bacillus* and *Clostridium.* Survival of these microorganisms was examined in model processed cheese samples supplemented with monoacylglycerols. In processed cheese samples, monoacylglycerols of undecanoic, undecenoic and adamantane−1−carboxylic acid supressed or prevented the growth of spore-forming bacteria for 140 days of storage. The most efficient monoacylglycerol in processed cheese samples was monoacylglycerol of adamantane-1-carboxylic acid which caused a notable reduction in microbial counts of *Bacillus cereus, Bacillus subtilis* and *Clostridium butyricum.*

Keywords: monoacylglycerol, antimicrobial activity, spore-forming bacteria, processed cheese, microbiology of processed cheese

ABSTRAKT

Zabezpečení kvality a zdravotní nezávadnosti potravin je hlavním cílem potravinářské mikrobiologie. Jedním z možných přístupů je snaha potlačit růst a množení nežádoucích mikroorganizmů přímo v potravinách. Přídavkem antimikrobik lze účinně blokovat či zpomalit růst kontaminující mikroflóry a prodloužit tak údržnost potravinového výrobku. Za tímto účelem jsou neustále hledány nové látky, které by mohly působit inhibičně na mikroorganizmy. Monoacylglyceroly jsou látky běžně se vyskytující v živočišných produktech s inhibičním účinkem vůči širokému spektru mikroorganizmů. Tyto látky by navíc díky svým emulgačním vlastnostem mohly i zlepšovat některé vlastnosti potravin.

Předkládaná dizertační práce je zaměřena na studium antimikrobní aktivity monoacylglycerolů vůči mikroorganizmům, které mohou kontaminovat potraviny. Pro testování inhibičních účinků *in vitro* bylo připraveno sedm monoacylglycerolů lišících se povahou kyseliny vázané na glycerol. Zvoleny byly monoacylglyceroly mastných kyselin se sudým i lichým počtem uhlíků, monoacylglyceroly nenasycených mastných kyselin a monoacylglyceroly připravené z netradičních kyselin. Na základě výsledků studia antimikrobní aktivity monoacylglycerolů v podmínkách *in vitro* lze konstatovat, že inhibiční účinek těchto látek závisí na charakteru esterifikované kyseliny. Testované mikroorganizmy byly citlivé zejména k působení monoacylglycerolů kyselin s 10, 11 a 12 uhlíky v řetězci. Kromě snížení hustoty mikrobiální populace vedla aplikace monoacylglycerolů také k prodloužení lag-fáze a snížení specifické růstové rychlosti studovaných kmenů. Podle experimentálních výsledků získaných *in vitro* byly vybrány čtyři monoacylglyceroly, jejichž vliv na růst mikroorganizmů byl sledován ve vzorcích tavených sýrů. Vzhledem k tomu, že sporulující bakterie patří mezi nejzávažnější kontaminanty tavených sýrů, byly modelové vzorky sýrů zaočkovány právě sporulujícími bakteriemi rodu *Bacillus* a *Clostridium.* Růst těchto bakterií v tavených sýrech s přídavkem monoacylglycerolů byl sledován po dobu 140 dnů. Přídavek monoacylglycerolu kyseliny undekanové, undecenové a adamantan-1-karboxylové vedl k redukci či úplné inhibici růstu sporulujících bakterií v tavených sýrech. Nejlepších výsledků bylo však dosaženo s netradičním monoacylglycerolem adamantan−1−karboxylové kyseliny, jehož přítomnost v tavených sýrech bránila růstu bakterií *Bacillus cereus*, *Bacillus subtilis* a *Clostridium butyricum.*

Klíčová slova: monoacylglycerol, antimikrobní aktivita, sporulující bakterie, tavené sýry, mikrobiologie tavených sýrů

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LIST OF ABBREVIATIONS

AAS	atomic absorption spectroscopy
ADI	acceptable daily intact
CFU	colony forming units
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohaemorrhagic strains of *E. coli*
ES	emulsifying salts
GC-MS	gas chromatography-mass spectrometry
GI	growth index
GRAS	generally recognised as safe
HLB	hydrophilic/lipophilic balance
HPLC	high performance liquid chromatography
HSV-1	herpes simplex virus type 1
MAG(s)	monoacylglycerol(s)
MAG C8:0	monoacylglycerol of caprylic acid (monocaprylin)
MAG C9:0	monoacylglycerol of nonanoic acid
MAG C10:0	monoacylglycerol of capric acid (monocaprin)
MAG C11:0	monoacylglycerol of undecanoic acid
MAG C11:1	monoacylglycerol of undecenoic acid
MAG C12:0	monoacylglycerol of lauric acid (monolaurin)
MAG C14:0	monoacylgycerol of myristic acid
MAG C15:0	monoacylglycerol of pentadecanoic acid
MAG C16:0	monoacylglycerol of palmitic acid
MAG C18:1	monoacylglycerol of oleic acid
MAG ACA	monoacylglycerol of adamantane-1-carboxylic acid
MAG PFUNDA	monoacylglycerol of perfluoroundecanoic acid
MALT	mucosa-associated lymphoid tissue
MIC	minimum inhibitory concentration
NMR	nuclear magnetic resonance spectroscopy
OD	optical density
TLC	thin-layer chromatography
TSST-1	toxic shock syndrome toxin 1

1. LITERATURE REVIEW

In the environment of foodstuffs, many microorganisms may be proliferated. The consequences of undesirable microbial growth in foods are health risks due to the presence of pathogenic microorganisms and economic losses due to spoilage microorganisms. Preservation technologies are designed to protect foods from the effects of microorganisms and inherent deterioration. There are several approaches that may be used to preserve foods. Microorganisms in foods may be inhibited or inactivated by physical methods (e.g., heat, cold, reduced water activity) or through application of antimicrobial compounds.

Antimicrobial compounds continue to be one of the most important classes of food additives. 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 international regulatory agencies are generally very strict as to requirements for toxicological evaluation of novel direct food antimicrobials. Finally, there is a current worldwide drive for a healthier lifestyle, which has led to a rising demand for fresh foods, free from “chemical additives”. On the one hand, the word preservative on food product labels seems to evoke a negative reaction from many consumers. On the other hand, consumers expect foods to be readily available year-round, and to have a reasonably long shelf life.

One group of antimicrobial compounds found in nature and considered to have little or no toxicity is the fatty acids and their corresponding esters. These compounds include monoacylglycerols that are widely used for their emulsifying abilities in the food processing industry. Monoacylglycerols seem 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.

The presented thesis deals mainly with the antimicrobial activity of monoacylglycerols. Seven monoacylglycerols differing in the structure of acid bound have been prepared for testing their potential antimicrobial effect *in vitro* and those with the widest antimicrobial spectrum were evaluated for their action in processed cheese samples.

1.1. MONOACYLGLYCEROLS

Monoacylglycerols (MAGs) are partial esters of the trihydric alcohol glycerol in which only one of the hydroxyl groups is esterified with a fatty acid. Monoacylglycerols (formerly called monoglycerides) exist in two forms depending on whether the primary (α) or secondary (β) hydroxyl is acylated.

α–monoacylglycerol has a single fatty acid esterified to *sn*-1 or *sn*-3 (primary) positions of the glycerol backbone. The β-isomer has the fatty acid esterified at the *sn*-2 (secondary) position [1, 2].

Physical and chemical properties are influenced by the nature of fatty acid carbon chain. The melting temperature of MAG increases with increasing number of carbon atoms and decreases with increase in unsaturation [3-5]. The appearance of monoacylglycerols varies from pale straw to brown coloured oily liquid to a white or slightly off-white waxy solid. The solid may be in the form of flakes, powders or small beads. Monoacylglycerols are insoluble in water but can form stable hydrated dispersions. MAGs are polymorphic and can exist in different crystal forms depending on the temperature [6-8].

The key molecular characteristic of a monoacylglycerol is that it is amphiphilic in nature, with the lipophilic (or hydrophobic) part of the molecule which has high affinity for lipid (nonpolar) environment and the hydrophilic part preferring to be in an aqueous (polar) environment. Amphiphilic properties of MAGs explain their well-known use as emulsifiers in margarines and other water in oil (w/o) emulsions [2, 5, 9, 10]. Emulsifier molecules adsorb to oil-water interfaces, which minimizes the contact area between hydrophilic and hydrophobic regions and therefore reduces the interfacial tension. This reduction is important during homogenization because it facilitates further disruption of emulsion droplets and provides a repulsive force to prevent the droplet aggregation [10]. Monoacylglycerols are very useful in food emulsions because they are non-ionic and not extremely sensitive to acid or base conditions. Optimal emulsification is usually achieved by combining MAGs with co-emulsifiers [11, 12].

Several methods have been developed to classify emulsifiers; the hydrophilic/lipophilic balance concept (HLB) is the most widely used. The HLB scale is based on the relative percentage of hydrophilic to lipophilic groups in the surfactant molecule and gives an indication of the relative affinity of a surfactant molecule for the oil and aqueous phases [10, 13]. Monoacylglycerols possess a lipophilic character and are therefore assigned with a low HLB number (3-6) [8].

Mono- and diacylglycerols may be present as minor components of oils and fats, either as intermediates in the biosynthetic pathway or as a product of partial lipolysis. MAGs are formed in the intestine during digestion of fat and are absorbed and transported before being reconverted to triacylglycerols for transport through the blood lipoproteins [1]. MAGs are also commonly present in bovine milk at small amounts as minor lipids along with phospholipids, diacylglycerols and glycolipids [14].

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. Mono- and

diacylglycerols have no limitation on the acceptable daily intact (ADI) value and can be added to foods *quantum satis.* Thus, their record of safety allows the application of monoacylglycerols to foods with great confidence [8].

1.1.1. Main applications of monoacylglycerols

The first mono- and diacylglycerols were synthesised in 1853 by the Frenchman Berthelot. The major breakthrough, however, was the application of MAGs in the 1930s in the margarine industry on a large scale. In 1936, the use of mono- and diacylglycerols was patented for ice cream applications. Nowadays, the total world production of emulsifiers is estimated to be in the order of 300,000 metric tons. This includes approximately 20 different types of emulsifiers. Mono- and diacylglycerols and their derivatives account for about 70% of the world production of food emulsifiers and therefore are considered as the most important group [8].

Besides functioning as surfactants in emulsions, MAGs are also utilized to modify starch or protein-containing products via complex formation with amylose or proteins, or to modify physical characteristics of fats by controlling fat crystal polymorphism. Thus, their functionality is dependent not only on their surfactant properties but also on physical characteristics such as crystalline behaviour, solubility in oils and fats, and interactions with water [6, 11].

The major applications in the food industry are typically in fat-based products, such as margarine, spreads and bakery fats (shortenings) [8]. MAGs of stearic, palmitic, oleic and linoleic acids are incorporated into margarine as emulsifying agents in proportions of 0.2 to 0.3% by weight [4]. Typical table margarine is water in oil (w/o) emulsion with a fat content of 60-80%. The benefits of the addition of MAGs include fine distribution of water droplets in the fat phase, smooth spreading consistency, stable crystalline structure and pleasant melt-in-the-mouth sensation for the consumer. The benefits of MAG addition to low fat spreads with a fat content 35-45% are the same as in table margarines, but unsaturated MAGs are often used. Unsaturated MAGs have a higher surface tension reducing activity than saturated MAGs, therefore less number of molecules is required to stabilise fine water droplets in the oil phase [8].

The role of monoacylglycerols in ice cream is to reduce the oil/water interfacial tension more than milk protein does alone, the homogenisation process is made more effective and a narrower fat particle size distribution with a well-defined total surface area is thus obtained. The usual MAG dosage in ice cream is 0.15% [8].

MAGs are also widely used in bakery applications, ice creams, frozen desserts, chewing gum and chews. In bakery applications MAGs have favourable influence on rheological properties of the bread dough, increase the durability of bread and affect a solubility of the protein fraction [15, 16]. Breads containing monoacylglycerols possess an improved shelf life due to retarded staling rate. In

addition to their antistaling benefit, MAGs in bakery products result in reduction of interfacial tension, improved dispersion of ingredients and increased aeration [2]. Monoacylglycerols increase the fermentation stability of the dough, i.e. fully fermented dough is resistant to collapse by mechanical shock during transport in bakeries via trays or belts from the proofing cabinet to the oven [8]. Besides fermentation stability, complexation of monoacylglycerol with starch or, more specifically, amylose, is of utmost importance in enhancing the shelf-life (crumb softness) of bread and cakes [4, 8, 17]. Interactions of monoacylglycerols with starch are also important in the production of dry pasta. Addition of hydrated monoacylglycerols to the pasta dough will result in less cooking loss and decreased stickiness in the finally cooked pasta [8].

Monoacylglycerols are also used as adjuvants for their lubricating and texturing properties in the production of potato flakes as well as in pre-cooked rice [4].

Due to their crystal polymorphism and similarity to triacylglycerols, MAGs can be used as fat extenders for low-fat dairy applications, providing better melting characteristics than non-lipid fat replacers [18].

In cosmetic applications, MAGs of saturated fatty acids are commonly used. The features of interest of these MAGs include the stability to oxidation, chemical inertness in the presence of active substances, emulsifying and emollient action, water dispersing activity and penetrating action [4]. Monoacylglycerols are part of the composition of natural lipids of the skin, thus possess high compatibility with skin and mucosa. Incorporation of MAGs in cosmetics and body care products does not appear to have any contra-indication or irritating action [4, 19]. Furthermore, their compatibility with skin helps improve the feel of various skin care products. Besides the improved skin feel they induce, they also reduce defatting of the skin possibly caused by surfactant-based cleansers [20]. MAGs find the widest use in the formulation of ointments and lotions for their ability to stabilize emulsions. Other products, where MAGs are applied, are hair-colouring and hair-setting products [4].

MAGs find applications also in pharmaceutical industry. Monoacylglycerol molecules spontaneously self-assemble into various liquid crystalline structures when present in an aqueous environment. The various phases can be used to achieve different functionalities, e.g. to protect molecules from chemical degradation, to solubilize drugs and nutrients or to control the release of drugs [21]. Due to these properties, MAGs can be employed as vectors for active substances. Moreover they are able to modify the permeability and composition of cell membranes and even if their target is intracellular, the interaction with this barrier plays a fundamental role [22-25].

A number of medium-chain saturated fatty acids and their monoacylglycerols have been suggested as components of antimicrobial hydrogel formulations for the purpose of preventing transmission of pathogens to mucosal membranes, particularly sexually transmitted viruses, such as herpes simplex virus and

human immunodeficiency virus, and bacteria, such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [26-29].
Another interesting possibility is the use of monoacylglycerol/fatty acid suspensions as immunological adjuvants that can enhance the immunogenicity of antigen formulations [30].
Due to plastifying, lubricant and antistatic properties, MAGs are employed in plastics industry, for example in the manufacture of methacrylic resins. In the fibre and textile industry MAGs are utilized as aqueous emulsions with anti-static properties, especially in the manufacture of polyolefin fibres. They improve water- and oil-repellent behaviour and enhance colour-fastness and contrast in fabrics [11].

1.1.2. Methods for production of monoacylglycerols

Monoacylglycerols have been produced on an industrial scale since 1960 by glycerolysis of triacylglycerols. More recently, MAGs have been produced by chemical synthesis using a variety of substrates and reagents including glycerol and its derivatives. Depending on the starting substrate, MAGs may be prepared by [4]:

- direct esterification of fatty acids by glycerol
- interesterification of triacylglycerols with glycerol (glycerolysis)
- transesterification of methyl esters of fatty acids with glycerol
- hydrolysis of fats and oils
- condensation of fatty acids with glycidol or its derivatives

The two most prevalent commercial preparations of mono- and diacylglycerols are direct esterification of glycerol with fatty acids and glycerolysis of natural or hydrogenated fats and oils. Both processes yield approximately the same equilibrium distribution of mono-, di- and triacylglycerols. The glycerolysis procedure is more economical because fats are cheaper than fatty acids and less glycerol is required [2].
Direct esterification of glycerol with fatty acids is carried out at a very high temperature 200 - 250 °C in the presence of alkaline catalyst, usually sodium hydroxide [8]. The ratio of glycerol to fatty acids determines the concentration of mono-, di- and triacylglycerols in the final product. Higher levels of glycerol produce higher concentrations of monoacylglycerols. Water is continuously removed by distillation, causing the equilibrium to shift toward products. Progress of the reaction is monitored by periodic measurement of the acid value. When the reaction is complete, the catalyst is neutralized to stop equilibration, and excess glycerol is removed by distillation at reduced pressure [2]. This process gives rise to fractions containing 40 - 56% of 1-monoacylglycerols. The concentration of 1-MAG can be raised to at least 90% by molecular distillation. The main disadvantage of this process is the use of harsh conditions (high

temperature, high pressure and inert atmosphere), the nature of the catalyst and poor selectivity which leads to a relatively low yield of monoacylglycerol [4].

Glycerolysis is the reaction of triacylglycerols with glycerol at high temperatures (180 - 220 °C) in the presence of an inorganic catalyst under a nitrogen gas atmosphere [11]. Higher glycerol/fat ratios require higher reaction temperatures to force the reaction to completion [2]. The heat and catalyst promote a transfer of acyl chains from triacylglycerols to glycerol [11]. Alkaline catalyst, such as calcium hydroxides are often used. Since the reaction is carried out at high temperatures, side reactions can produce dark colours and off flavours [2]. The product is a mixture of mono- and diacylglycerols and unreacted or re-esterified triacylglycerol. Molecular distillation is used to separate MAGs into a more pure form. MAGs are evaporated under high vacuum while the remaining di- and triacylglycerols are recycled for further glycerolysis [11]. The process is very energy consuming because of the high reaction temperature and the distillation, therefore increasing attention is paid to enzymatic glycerolysis. Lipase-catalysed enzymatic synthesis is performed at milder reaction conditions (especially lower temperatures 30 - 70 °C) allowing a wider range of fat or oil reactants to be used. Other advantages include less energy input, a possibility of the choice of acyl-specific lipases to yield acyl-specific products, and a more environmentally friendly process. The major disadvantages are reduced yields relative to glycerolysis, and in some cases the use of solvents necessitating a removal step [11, 31, 32].

In the last decade, many approaches have been investigated in the enzymatic synthesis of MAGs. Except for glycerolysis, these are direct esterification of fatty acids, transesterification or hydrolysis of triacylglycerols [4, 32]. To obtain MAG by enzymatic hydrolysis, it is necessary to control the reaction so that complete hydrolysis is avoided. Application of specific lipases is possible, e.g. 1,3-*sn*-specific lipase yielding 2-MAG. The main problem in this type of reaction is the low overall yields of MAG [32]. The discovery of high stability of lipases in organic solvents offered the possibility of a reverse reaction of hydrolysis - esterification using free fatty acids or transesterification using esters. For that purpose conditions are needed in which the enzyme will catalyse the synthesis reaction rather than the hydrolysis. Most important, low water content and low water activity is necessary [33]. Although these lipase-catalysed reactions are promising, the achieved conversions and selectivities differ significantly depending on the source of lipase, the reaction system, and other parameters. Therefore a prediction of these reactions with respect to substrate specificity, time and yield, is still difficult [32]. Other problems with the process are high cost and denaturation of the enzyme as well as slow reaction times [2].

Another interesting pathway to pure 1-MAGs is offered by a nucleophile opening of the epoxide ring of glycidol (2,3-epoxy-1-propanol). The mentioned procedure is universal and makes the preparation of virtually any 1-MAG

possible. When an optically active glycidol is used, enantiomers of chiral monoesters can be obtained [34]. The regioselectivity of the epoxide ring opening depends on the reaction conditions as well as on the type of catalyst used [35]. The nucleophilic epoxide ring opening may be catalysed by titanium isopropoxide, however, the reaction yield is low due both to formation of by-products and to difficulties in separation linked to the use of large amounts of titanium salts [36]. Other possibilities involve the use of tertiary amines or ammonium salts [37-39].

High regioselectivity of glycidol-fatty acid reaction can be achieved using catalysts based on chromium (III) compounds, especially chromium (III) acetate. In the reaction process a coordination bond between chromium (III) ion and the oxygen atom of the oxiran ring is formed and subsequently, the acetate ion attacks the less substituted carbon atom of oxiran [40]. Janiš *et al*. [34, 41] employed chromium (III) complexes of various fatty acids as catalysts for the reactions of fatty acids and glycidol. With mild reaction conditions and short reaction time, as high conversions as 90-95% were achieved and no by-products were detected in the reaction mixture. In a later study, commercial chromium (III) acetate hydroxide was also successfully used. After the crystallization of crude reaction product from ethanol, the purity of acquired 1-MAGs was very high such that it can be regarded as a chromatographic standard [35].

Reactions of fatty acids with glycidol can be carried out in the absence of solvent if the starting fatty acid is liquid or possesses a low melting point. For fatty acids with high melting point the reaction has to be conducted in a suitable solvent and the removal of the solvent from reaction product is therefore necessary [35].

Limitation for the use of Cr (III) compounds for catalysis of glycidol-fatty acid reaction may arise from contamination of the reaction product with chromium. This fact could restrict the possibility of its potential application, for example, in the food industry or pharmaceutical industry. By crystallization of crude reaction products from appropriate solvents, the chromium content can be reduced significantly [35].

1.2. ANTIMICROBIAL ACTIVITY OF MONOACYLGLYCEROLS

Fatty acids and their derivatives have a long and respected historical record for having antimicrobial activity. Salts of fatty acids (soaps) have been used for hundreds of years as cleaning and disinfecting agents. However, research on antimicrobial properties of fatty acids only began in the 1930s. There is a connection between dietary fat and resistance to infectious diseases, but not until 1970s did researchers show that the antimicrobial action of milk fat depends on the presence of lipases, that releases free fatty acids and monoacylglycerols [12, 42].

Many authors have discussed the antimicrobial effects of fatty acids. In an attempt to bring some order to the vast amount of literature on the antimicrobial activity of fatty acids and derivatives, a number of generalizations have been suggested by Kabara and his co-workers [43-46]:

- except for short-chain fatty acids with fewer than 8 carbon atoms, fatty acids do not affect gram-negative bacteria,
- the most active saturated fatty acid is lauric acid (C12), the most active monounsaturated fatty acid is palmitoleic acid (C16:1), and linoleic acid (C18:2) is the most active polyunsaturated acid,
- position and number of double bonds are more important to long-chain (>C12) than shorter chain fatty acids.

It was also found, that fatty acids esterified to monohydric alcohols, such as ethanol and methanol, are inactive against microorganisms, but esterification to polyols increases their activity. From these results it was concluded that some hydrophilic group was necessary for biological activity. One of the more common polyhydric alcohols, glycerol, was esterified and found to be more active than the corresponding free fatty acids. These findings were published and confirmed by many authors [45-48].

Antimicrobial activity of MAGs as well as their other functional properties depends on the nature of fatty acid esterified to glycerol backbone, more precisely on the number of carbon atoms and on the presence of double bonds in the fatty acid chain [43, 45, 49]. MAGs of fatty acids with short or medium chain length (>C12) are stronger antibacterial agents than those with longer chain. Decreasing effectiveness of MAGs with longer chain may be related to increased hydrophobicity and decreased solubility [50]. Differences in antimicrobial action were also found in different positional isomers. Conley and Kabara [45] found lower antimicrobial effect in *sn*-2 isomer of MAG of lauric acid.

The exact mechanism by which fatty acids and their monoacylglycerols are able to kill or inhibit the growth of microorganisms is not known, however, numerous hypotheses have been suggested to explain the general mode of antimicrobial action of free fatty acids and MAGs. It seems that unlike antibiotics, fatty acids and their esters have several modes of action that are nonspecific. Thus, development of resistance to these compounds has a very low frequency and it is generally considered as very unlikely [12, 29, 51]. Based on results of electron microscopic studies monoacylglycerols are proposed to act as non-ionic surfactants that penetrate and get incorporated into bacterial plasma membrane [52]. These compounds resemble the bipolar membrane of the bacterial cell due to having both a hydrophilic head and hydrophobic tail. This similarity suggests that fatty acid esters target the bacterial and fungal cell membrane, thus killing by penetrating and disrupting normal function of cell membranes [53]. Damage to cell membrane usually results in the release of

intracellular constituents, the first being potassium (K^+) leakage followed by inorganic phosphates (P_i), amino acids and then larger molecular weight material indicative of gross injury [12]. Thompson *et al.* [54] found that the inhibitory effects were associated with the incorporation of fatty acids into bacterial membranes followed by development of abnormal cell forms and cell lysis. Another school of thought hypothesizes that short and medium fatty acids diffuse into bacterial cells in their undissociated form and dissociate within the cytoplasm, thereby leading to intracellular acidification with consequent inactivation of intracellular enzymes and inhibition of amino acid transport [55-57].

Antimicrobial action of MAGs may be affected by the presence of other compounds. Proteins, especially lipophilic proteins such as albumin, and other nutrients, including fat and starch, interact with fatty acids and MAGs. Lipid-protein complexes can be formed in the presence of proteins and therefore bactericidal activity of surface active agents like MAGs is markedly reduced [12, 50, 51]. On the other hand, the antibacterial activity of MAGs has been shown to be enhanced when combined with high temperatures [58], freezing, acidulants [57, 59, 60], chelating agents, nisin [61] or lactoperoxidase system [62].

Many articles have also been published recently on the antimicrobial activity of MAGs. Monoacylglycerols of various fatty acids have showed promising activity against diverse microorganisms including gram-positive and gram-negative bacteria [43, 63-65], spore-forming bacteria [61, 66], yeasts [52, 65], filamentous fungi [65, 67, 68] and also enveloped viruses [26, 69]. The literature review of antimicrobial action of monoacylglycerols is shown below.

1.2.1. Antimicrobial activity of monoacylglycerols against gram-positive bacteria

There are numerous studies dealing with the antimicrobial effect of the individual MAG against gram-positive bacteria, however not many of them compare the activity of several MAGs under the same conditions. One of the few is the study published by Buňková *et al.* [49]. The authors compared the inhibitory effects of seven 1-MAGs on selected gram-positive species participating on food spoilage or posing a risk to the consumer´s health. Monoacylglycerols of fatty acids with 8 - 16 carbon atoms were chosen for these purposes. It was concluded, that the gram-positive food-borne pathogens and spoilage bacteria were sensitive mainly to 1-MAGs containing fatty acids with 10 - 14 carbons in chain; with MAG of lauric (MAG C12:0), undecanoic (MAG C11:0) and capric (MAG C10:0) acid being the most efficient. For these monoacylglycerols minimum inhibitory concentrations (MIC) in five gram-positive species ranged from 25 to 250 µg/ml. Similar MIC values were reached in the study of Růžička *et al.* [65]. Their results showed, that MAG C10:0 at

concentrations of 100 - 250 µg/ml was able to completely stop the growth of all gram-positive bacteria tested as well as MAG of lauric acid (MAG C12:0) with levels of MIC from 20 to 200 µg/ml. In both studies, authors also pointed out the differences in sensitivity to MAGs in different gram-positive species.

Among monoacylglycerols MAG of capric (MAG C10:0) and MAG of lauric (MAG C12:0) acid seem to be the most efficient in killing gram-positive bacteria. As early as in 1970s the antibacterial action of both these MAGs was proved against beta-hemolytic streptococci, *Streptococcus pneumoniae, Staphylococcus aureus*, *Staphylococcus epidermidis* and micrococci [43, 45]. Schlievert *et al.* [70] demonstrated that MAG C12:0 was inhibitory to the growth of staphylococci and streptococci at relatively low concentrations 10 – 20 µg/ml. Moreover, the production of exoproteins by both staphylococci and streptococci was also inhibited by MAG C12:0 concentrations that did not inhibit bacterial growth. The production of TSST-1 (toxic shock syndrome toxin 1) by *Staphylococcus aureus* was stopped by the MAG C12:0 and authors discussed the potential use of this compound to reduce risk of menstrual toxic shock syndrome and related illnesses. Activity of MAG C12:0 can be enhanced by the addition of other antimicrobial compound. Wakabayashi *et al.* [71] discovered that a combination of MAG C12:0 with lactoferricin B killed *S. aureus* more rapidly than either agent alone. Bacterial mastitis pathogens, including *Streptococcus agalactiae, Str. dysgalactiae* and *Str. uberis* were shown to be very sensitive to MAG of caprylic acid (MAG C8:0) and the presence of this MAG resulted in a rapid decline in the populations of streptococci after 1 minute of treatment [72].

Enterococci are gram-positive cocci with relatively high resistance to environmental conditions and antimicrobials. They can grow at 10 to 45 °C, in media with high salt concentrations, and in environments with broad pH values. In addition, enterococci have the capacity to acquire a wide variety of antimicrobial resistance factors and can survive some types of food processing. They have been implicated in outbreaks of food-borne illnesses and in the spoilage of processed cooked meat, raw meat, milk and dairy products [73, 74]. In *Enterococcus faecalis,* growth inhibition was reported after the application of MAG C12:0 at concentration of 100 µg/ml and at 250 µg/ml when MAGs with 10, 11 and 14 carbon atoms in fatty acid chain were used [49]. Similar MIC values were recorded by Růžička *et al.* [65] for MAG C10:0 and MAG C12:0.

Very low MIC of MAG C12:0 was recorded for another gram-positive species, a pathogenic bacterium that contaminates foods *Listeria monocytogenes.* According to the results of Bal´a and Marshall [75] eight strains of *L. monocytogenes* were inhibited by 16 µg/ml of MAG C12:0 on commercial media. MAG activity was reduced on catfish-based medium and approximately four- to eightfold more MAG was required to inhibit the test strains due to neutralization of antimicrobial activity by food polymers. Efficiency of MAG C12:0 against *L. monocytogenes* was confirmed by Blaszyk and Holley [76],

who suggested increasing the activity of MAG by combining with eugenol and sodium citrate. Wang and Johnson [50] found that in brain heart infusion broth MAG C12:0 was bactericidal to *L. monocytogenes* at 10 µg/ml, whereas MAG of myristic (MAG C14:0) and MAG of palmitic (MAG C16:0) acid were not inhibitory at 200 µg/ml. The bactericidal activity of MAGs was higher at pH 5 than at pH 6. MAG of lauric acid inactivated *L. monocytogenes* in skim milk, but was not inhibitory in whole milk because of higher fat content. The activity of MAGs can be affected by the growth phase. The study of Chavant *et al.* [77] was designed to investigate the effect of sanitizers on survival of planktonic or sessile *L. monocytogenes* cells at different phase of growth. MAG of lauric acid had a more significant impact on stationary cells than on exponential cells in the planktonic mode of growth.

There are also reports on antibacterial activity of monoacylglycerols on lactic acid bacteria. Blaszyk and Holley [76] showed that MAG of lauric acid either alone or combined with eugenol and sodium citrate inhibited the growth of *Lactobacillus sake, Lb. curvatus* and *Leuconostoc mesenteroides.*

1.2.2. Antimicrobial activity of monoacylglycerols against gram-negative bacteria

According to various literature sources, monoacylglycerols are less effective in inhibiting the growth of gram-negative bacterial species when compared with gram-positive bacteria [65, 78]. Higher resistance of gram-negative species is probably connected with differences in cell wall composition. Cell wall of gram-negative bacteria contains outer membrane with lipopolysaccharides that gives to the bacterium protective function. The effectiveness of fatty acids and monoacylglycerols against gram-negative bacteria seems to be species-dependent and varies even among different strains. As an example, Altieri *et al.* [63] in their study concluded, that MAG C12:0 performed a strong inhibition against *Escherichia coli* and *Yersinia enterocolitica*, but not against *Salmonella* sp., although all mentioned species belong to the same family *Enterobacteriaceae.*

Most studies dealing with the antimicrobial effect of MAGs against gram-negative bacteria are focused on solely two monoacylglycerols - MAG of lauric (MAG C12:0) and MAG of capric (MAG C10:0) acid. Buňková *et al.* [49] examined antimicrobial action of seven different MAGs against gram-negative bacteria. Within monoacylglycerols tested, MAG C10:0 had the strongest activity on gram-negative species. In this MAG, the growth of *Citrobacter freundii, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* Enteritidis was not detected at the presence of MAG at 1500 µg/ml. Significant reduction in the growth of these bacteria also occurred after the addition of MAG C8:0 at concentrations higher than 500 µg/ml. Other monoacylglycerols including MAG

C11:0, MAG C11:1, MAG C12, MAG C14:0 and MAG C16:0 caused only slight decrease in growth of gram-negative bacteria.
MAGs with odd number of carbon atoms - MAG C11:0 and MAG C11:1, were the subject of investigation of Doležalová *et al.* [79]. Compared to MAG C11:0, the unsaturated MAG exhibited more prominent antimicrobial properties against gram-negative bacteria. Nevertheless the satisfactory inhibition occurred only at relatively high concentrations. MAG of caprylic (MAG C8:0) acid was involved into a study dealing with antimicrobial evaluation of fatty acid esters and ether derivatives. The authors concluded that MAG C8:0 showed inhibitory activity against *E. coli* and had wider activity than MAG C12:0 [48].
Another gram-negative bacterium *Helicobacter pylori* was sensitive to several medium chain monoacylglycerols. *Helicobacter pylori* is firmly established as the etiologic agent of acute or chronic gastritis and a predisposing factor in peptic ulcer disease, gastric carcinoma and B-cell mucosa-associated lymphoid tissue (MALT) lymphoma [80]. Especially MAG C10:0 and MAG C12:0 proved to be active against *H. pylori* even after very short incubation times [19]. Incubation of *H. pylori* with saturated MAGs ranging in carbon chain length from 10 to 14 caused a 4-log-unit or greater reduction in the number of viable bacteria after exposure for 1 h. Lower levels of bactericidal activity were observed with MAG C9:0, MAG C15:0 and MAG C16:0. Higher levels of monoacylglycerols were required for inhibitory activity in the presence of proteins [51].
Medically important gram-negative coccus *Neisseria gonorrhoeae* which causes gonorrhoea is effectively killed by exposure for 1 minute to MAG C10:0. This rapid *in vitro* killing is an essential prerequisite for the possible use of MAG in the prevention of sexually transmitted diseases [29]. Due to high *in vitro* efficacy of MAG C10:0 against a number of sexually transmitted microbes such as *Chlamydia trachomatis* or herpes simplex virus type 1 (HSV-1), this lipid may be useful as a microbicidal agent for both treatment and prevention of sexually transmitted diseases [26, 28].
Inhibitory activity of MAGs against gram-negative bacteria can be enhanced by other factors, e.g. decrease in pH of the environment [81] or by the addition of other inhibitory agents such as eugenol [76], chelating agents [82] or lactoperoxidase systems [62]. In experiments of Razavirohani and Griffiths [83, 84] MAG of lauric acid was effective against all gram-positive bacteria studied, but was only effective against gram-negative bacteria in the presence of ethylenediaminetetraacetic acid (EDTA).

1.2.3. Antimicrobial activity of monoacylglycerols against spore-forming bacteria

Spore-forming bacteria and bacterial endospores constitute a target microbial group of great concern in food preservation. This group contains some of the

most pathogenic and most heat resistant microorganisms that contaminate foods. Bacterial spores are highly resistant to pasteurization treatments and hence, subsequent spore germination and growth of vegetative cells can occur. At least two gram-positive spore-forming genera are known to cause bacterial food poisoning - *Clostridium* and *Bacillus* [85].

According to Ababouch *et al.* [86, 87] fatty acids and MAG of lauric acid (MAG C12:0) were able to inhibit the process of spore germination and outgrowth in *Bacillus cereus*.

Bacterial endospore germination has been defined as the degradation process by which the dormant state is irreversibly terminated. Germination is followed by an outgrowth which is the process of synthesis of new bacterial macromolecules and conversion of germinated spores into a newly emerged vegetative cell [88].

The mechanism of outgrowth inhibition by MAG was suggested as an inhibition of oxygen consumption which indicates that the inner membrane and its enzymes responsible for oxygen transport are the possible sites of action. The activity of MAG C12:0 was also confirmed for *B. cereus* vegetative cells [86, 87]. These results were confirmed by Cotton and Marshall [89] who challenged vegetative cells of *B. cereus* with 25 µg/ml of MAG C12:0 prepared by two methods - water-dispersed with heat or dissolved in ethanol. Presence of MAG, regardless of preparation method, resulted in lower numbers of *B. cereus* cells compared to controls, but antimicrobial activity of MAG C12:0 was more pronounced when dissolved in ethanol than when heat-dispersed in an aqueous system.

Monoacylglycerols of fatty acids with odd number of carbons (MAG C11:0, MAG C11:1) has also been shown to supress the growth of *B. cereus* [79] as well as MAG C10:0 and MAG C14:0. Monoacylglycerols of fatty acids with 10 – 14 carbon atoms affect the growth of another species of the genus *Bacillus*, *Bacillus subtilis* [49]. Mansour *et al.* [61] investigated the effects of MAG C12:0 and nisin, alone or in combination, on *Bacillus licheniformis* spores in milk. MAG C12:0 and nisin acted synergistically on vegetative cells and outgrown spores, showing total inhibition at pH 6. MAG of lauric acid (MAG C12:0) had an inhibitory effect on *Bacillus anthracis Sterne*, grown from a live veterinary vaccine. This organism grew in a concentration of penicillin at 62 µg/ml, but was totally inhibited at the same concentration of MAG C12:0 [64].

The usefulness of MAG C12:0 in the heat inactivation of spores was substantiated by Kimsey *et al.* [90], who showed enhanced thermal inactivation of *Geobacillus stearothermophilus* in the presence of MAG C12:0. It was concluded that MAG of lauric acid could be used as food additive to reduce the heat treatment required to achieve commercial sterility of foods. This conclusion was supported by Chaibi *et al.* [66], who observed similar activity of MAG C12:0 in *B. cereus*.

Chaibi *et al.* [91] examined the inhibition of bacterial spores and vegetative cells by glycerol esters. Monoacylglycerols of myristic, linoleic, linolenic and lauric acid inhibited spores and vegetative cells of *Bacillus cereus*, *Clostridium sporogenes* and *Clostridium botulinum.* MAGs of palmitic, stearic and oleic acid had a partial effect and di- and triacylglycerols were ineffective. In general, *C. botulinum* and *C. sporogenes* were more resistant than *B. cereus*. Authors have also found that the concentrations of MAGs needed to inhibit spore germination and outgrowth were higher than the concentrations needed to inhibit cell multiplication.

1.2.4. Antimicrobial activity of monoacylglycerols against yeasts and filamentous fungi

The study of Růžička *et al.* [65] was aimed at assessing the effects exhibited by two monoacylglycerols (MAG C10:0, MAG C12:0) against filamentous fungi and yeast-like organisms. Determined MIC values revealed the sensitivity of *Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Aureobasidium pullulans, Candida tropicalis* and *Candida albicans* to MAG C10:0 at concentration ranges from 150 to 200 µg/ml. The inhibitory activity of MAG C10 against *Candida albicans* was described also in works of Kabara *et al.* [43] and Bergsson *et al.* [52]. In the latter study capric acid and its 1-MAG caused the fast and effective killing of three strains of *C. albicans* leaving the cytoplasm disorganized and shrunken because of a disrupted plasma membrane.

Microbistatic or microbicidal effects of MAG C11:0 and MAG C11:1 against *C. albicans* and *S. cerevisiae* were documented and published by Doležalová *et al.* [79]. Both MAGs had microbistatic effects against yeasts at concentration of 250 µg/ml and showed microbicidal effect at concentrations not exceeding 400 µg/ml. Inhibitory activity of these MAGs was also studied on several species of micromycets e.g. *Alternaria* sp., *Aspergillus niger*, *Mucor racemosus* or *Cladosporium* sp. Up to 1000 µg/ml neither MAG showed total inhibition activity against any of the studied fungi, but the growth was notably reduced. In addition, authors noticed that MAGs in agar culture medium influenced not only colony size but also their macroscopic morphology and exopigment production.

Lisker and Paster [92] carried out extensive studies with MAG C12:0 and food-grade preservatives such as sorbic or propionic acid. MAG of lauric acid was highly efficient against several fungi genera including *Aspergillus, Penicillium, Fusarium, Rhizopus and Mucor* and in some species was even more active than sorbic and propionic acid. On the other hand, some authors reported only weak inhibition after application of MAG C12:0 on *Aspergillus* and *Penicillium* strains [65, 68]. It seems that fungal sensitivity to MAGs is not uniform even in the same fungi species and could vary from strain to strain.

Buňková *et al.* [68] carried out an evaluation of antifungal activity of MAGs with 8, 10 and 12 carbon atoms using micromycetes originally isolated from

contaminated bread and micromycetes from a collection of microorganisms. Results showed, that MAG C8:0 and MAG C10:0 at a concentration of 65 µg/ml possessed a very strong inhibition effect *in vitro* against *Penicillium piceum, P. chrysogenum, P. roqueforti* and *Alternaria* sp. MAG of lauric acid turned out to be the least efficient. Authors noticed that, *Monascus ruber* and *Aspergillus niger* were the most resistant species in the test. In the further part of the experiment MAGs were applied on the surface of freshly baked bread and prevented its deterioration for at least 14 days. Authors suggested using monoacylglycerols solutions for the protection of bread loaf surfaces as the suitable procedure for bakery products with a prolonged storage period.

MAGs with longer fatty acid chain (MAG C12 - C14) were examined for antifungal effectiveness against *Fusarium oxysporum* and *F. avenaceum* that belong to the most common contaminants of cheese surface. MAG C12:0 inhibited the growth of *F. avenaceum* within 30 days, but only reduced the colony diameter in *F. oxysporum.* MAGs of myristic and palmitic acid showed a moderate antifungal effectiveness [67].

Filamentous fungi (micromycetes) can be not only responsible for the deterioration of food products, but the formation of mycotoxins and other harmful metabolites often occurs even before the fungal contamination is visually noticed. Mansour *et al.* [93] found that MAG C12:0 was capable of significantly reducing the growth and aflatoxin production in aspergilli.

1.3. PROCESSED CHEESE PRODUCTS

Cheeses are an important component of the diet in the developed countries. Processed cheese belong to the newer branches within the area of dairy and cheese industry and their history dates back no more than 100 years ago. Originally, processed cheese was developed as a way of increasing the shelf life of cheese and improving the palatability of lower quality cheese [94].

Despite this fact, processed cheese belong to the most popular and worldwide commodities. The reason lies mainly in easy use, relatively good shelf-life and various possibilities of optimizing production costs. The growing worldwide production of processed cheeses is also enabled and supported by low cost relative to natural cheese due to incorporation of low-grade natural cheese, off-cuts and cheaper non-cheese milk solids, e.g. skim milk powder, whey, casein and caseinates.

From the perspective of consumer, popularity of processed cheese is caused especially by great variability of these products. Processed cheese may be used as a table product with a spectrum of consistencies ranging from firm, elastic and sliceable to creamy, smooth and spreadable. In a heated form, processed cheese products are also used as an ingredient in several cookery applications, e.g. in toasted sandwiches, pasta dishes or au-gratin sauces. Processed cheese products offer wide variety in size and shape of consumer packaging, which

makes them attractive. And last but not least, there is a wide range of flavours and ingredients that can be used for the production of processed cheese [95, 96].
Processed cheese products can be classified according to various aspects with the fat content in dry matter being the most important. According to the Regulation of the Ministry of Agriculture of the Czech Republic No. 77/2003 processed cheese can be classified into two groups: high-fat processed cheese (at least 60% w/w fat-in-dry matter) and low-fat processed cheese (up to 30% w/w fat-in-dry matter).
More detailed classification that reflects permitted ingredients and compositional parameters can be found in Anglo-Saxon countries. According to Fox *et al.* [96] there are five categories of processed cheese products:

1. Pasteurized blended cheese
   permitted ingredients include cheese, cream, anhydrous milk, fat, dehydrated cream, water, salt, food-grade colours, spices and flavourings, sorbic acid or potassium/sodium sorbate at levels ≤0.2% w/w; sodium propionates at levels ≤0.3% w/w.
2. Pasteurized processed cheese
   permitted ingredients are as for pasteurized blended cheese, but with the following optional ingredients: emulsifying salts (sodium phosphates or sodium citrates) at a level of ≤3% w/w; food-grade organic acids (lactic, acetic or citric acid) at levels such that the pH of the finished product is not less than 5.3.
3. Pasteurized processed cheese foods
   ingredients are as for pasteurized processed cheese but with extra optional dairy ingredients (milk, cream, skim milk, buttermilk, cheese whey, whey proteins in wet or dehydrated form).
4. Pasteurized processed cheese spread
   ingredients are as for pasteurized processed cheese but with the following extra optional food-grade hydrocolloids (carob bean gum, guar gum, xanthan gum, gelatine, carboxymethylcellulose, carrageenan at levels <0.8% w/w) and food-grade sweetening agents (sugar, dextrose, corn syrup, glucose syrup, hydrolysed lactose).
5. Pasteurized cheese spread
   permitted ingredients are as for pasteurized processed cheese spread, but emulsifying salts are not permitted.

Pasteurized processed cheese has moisture limited to 1% higher than that of the natural cheese from which it is made and cannot exceed 43% moisture. Fat must meet the same standards as for the natural cheese and it must contain at least 47% fat-in-dry matter. Pasteurized processed cheese food must contain at least

51% cheese, no more than 44% moisture and at least 23% fat. Moisture content in processed cheese spreads may ranges from 44 to 60%, but must include at least 51% cheese and 20% milkfat [95, 97].
Except for above mentioned groups, we can come into contact with processed cheese imitations or processed cheese analogues. These are nonstandard products that include nondairy proteins or fats, although some include milk derivatives such as casein and whey products. For their production, caseinates, proteins of non-dairy origin, vegetable oils and emulsifying salts are mainly used. These products are frequently developed as low-cost alternatives. Most of all, they are applied within the field of gastronomy. From a legislative point of view, processed cheese analogues cannot be called “processed cheese” [97, 98].

1.3.1. Basic principles of processed cheese production

Processed cheese is defined by the Regulation of the Ministry of Agriculture of the Czech Republic (No. 77/2003) as cheese which has been treated by heat with the addition of emulsifying salts. Processed cheese are therefore 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 [95, 96].
The main ingredients for processed cheese production are natural cheeses. Processed cheese can be produced by heating a mixture of different kinds of natural cheeses, which can be at different stages of maturity. Even natural cheeses with mechanical or other defects may be included in the product composition; however the use of natural cheeses with microbiological defects should be avoided. Particularly natural cheeses contaminated by spore-forming microorganisms are not recommended for the production of processed cheese [99].
Other ingredients can include quark, dairy fat (e.g. butter, cream, anhydrous milk fat), drinking water, additives and emulsifying salts.
Emulsifying salts (ES) are salts with polyvalent anions and monovalent alkaline metals. The term “emulsifying salts” is a little misleading as they are not emulsifiers in the sense of surface active chemicals and they do not play direct role in creating fat droplet dispersion. Their main effect in this instance is to improve the emulsifying ability of caseins. Heating the mixture of ingredients without the use of emulsifying salts would result in extensive clumping and coalescence of fat globules and a less homogenous distribution of the fat and casein phase [100]. The ensuing separation of hydrophobic and hydrophilic phases increases with the fat content of the cheese and the heat-induced effects are the most severe in high-fat cheeses. Hence, application of heat and mechanical shear without the presence of emulsifying salts usually results in the formation of heterogenous mass which undergoes extensive oiling-off and moisture exudation during manufacture and, especially, on cooling. In the

presence of ES, heat and shear result in the formation of a smooth, homogenous, stable product. This transition is facilitated by the ES-induced partial hydration and solubilization of casein which emulsifies the dispersed droplets of free fat [101].

Caseins in cheese are amphiphilic in nature, which allow them to function as emulsifiers. In cheese calcium is precipitated onto the casein matrix, reduces casein solubility and prevents caseins from using their emulsifying function. The ability to sequester calcium is one of the most important functions of emulsifying salts. This sequestration involves the exchange of the Ca^{2+} of the caseins for the Na^{+} of the ES. By means of exchanging calcium ions for sodium ions, more soluble sodium salts are formed from insoluble calcium caseinate. The increase in casein hydration is paralleled by a physical swelling of the caseinate dispersion and an increase in the viscosity of the melting processed cheese mass. The increase in viscosity is referred to as creaming in the processed cheese industry.

Apart from exchanging ions, ES are also used for pH adjustment. An appropriate pH value during processing affects protein conformation and hydration, calcium sequestration and textural and melting characteristics of the final product. Some phosphate-based emulsifying salts show buffering capacity and thus they are able to stabilise pH of the system against the surrounding effects [96]. Emulsifying salts also increase the water-holding capacity of proteins in processed cheese and improve the ability of caseins to emulsify fat, although the mechanism of this phenomenon caused by ES has not been fully elucidated [102, 103].

The most commonly used emulsifying salts are phosphate- or citrate-based salts. At the commercial level, ES are supplied increasingly as mixtures of different phosphates or of phosphates and citrates, which are made to impart certain functionalities (e.g. different degrees of meltability, sliceability, spreadability) to different products [102].

The selection of an adequate amount and type (or types) of ES is therefore essential factor in obtaining processed cheese with the desired functionalities, depending on the type, age and pH of the natural cheese and emulsifying conditions used [104].

The phosphate-based ES used in processed cheese manufacture are mainly the sodium salts of orthophosphates which contain one PO_4 group, and linear condensed phosphates such as pyrophosphates (two PO_4 groups), and polyphosphates (3 - 25 PO_4 groups). Potassium salts of phosphates can be also used as they give processed cheese with textural properties similar to those made with the equivalent sodium salts at similar concentration. However, they are rarely used in practice as they impart a bitter taste to the finished product, which becomes even more pronounced with storage [96, 105]. Within the citrates available, trisodium citrates are the most commonly used emulsifying agents. The presence of citrates in processed cheese is very similar to that of

orthophosphates and they are used mainly in mixtures with other ES, especially polyphosphates. Their main application lies in the field of block and sliced processed cheese.
Individual ES vary in their properties, such as calcium sequestration, buffering action, ability of casein hydration or ability of fat emulsification. According to their ability of calcium sequestration, emulsifying salts can be ranked in the following order: polyphosphates > pyrophosphates > orthophosphates > citrates [95, 96]. The effectiveness of different ES in promoting emulsification is in the following order: sodium tripolyphosphates > pyrophosphates > polyphosphates (P > 10) > orthophosphates > citrates [96]. Other properties of different emulsifying salts used in cheese processing are summarized in Table 1.

Table 1. *Emulsifying salts commonly used in pasteurized processed cheese products and their properties during processing [96]*

		Physico-chemical changes during processing			
		Calcium sequestration	**Buffering action**	**Casein hydration**	**Fat emulsification**
Citrates	Trisodium citrate	Low	High	Low	Low
Ortho-phosphates	Disodium phosphate Trisodium phosphate	Low	High	Low	Low
Pyro-phosphates	Disodium pyrophosphates Trisodium pyrophosphates Tetrasodium pyrophosphates	Medium	Medium	Very high	Very high
Poly-phosphates	Pentasodium tripolyphosphate Sodium tetrapolyphosphate Long-chain polyphosphates	High to very high	Low to very low	High to low	Very high to low

The manufacture of processed cheese involves the following major steps:

- formulation and selection of the different types and levels of ingredients,
- cleaning and comminution of the cheese,
- blending with water and other permitted ingredients,
- processing (heating, shearing) of the blend,
- homogenization of the molten blend,
- hot packaging and cooling.

Formulation involves selection of the correct type and quantity of natural cheeses, emulsifying salts, water and optional ingredients to get a product with

the desired composition, textural and functional properties. The type and degree of maturity of natural cheese, which is the major constituent of processed cheese, have a great influence on the consistency of the product. Cheese age, and hence a level of proteolysis, appears to be one of the most important selection criterions for blend formulation. Young cheese with 70 - 90% intact casein is required predominantly for the production of block processed cheese with good sliceability, whereas medium ripe cheese with lower level of intact casein is used for the production of cheese spreads [96, 103].

Cleaning generally involves the removal of surface contamination and rind. The subsequent processing needs an increase in the surface area of the cheese and increase homogenity of the formulated blend. Maximization of the surface area facilitates heat transfer within the blend and enables interactions between the cheese and other ingredients. For the size reduction the cheese is cut using hydraulically operated blades and minced by passing through high-speed shredders or mincing machines. The finally ground cheese is conveyed directly to the cooker where it is blended with emulsifying salts, water and other ingredients [95, 96].

Processing refers to the heat treatment of the blend, by direct or indirect steam, with constant agitation. Heat treatment has two main functions - to facilitate the physico-chemical changes which transform the blend to an end product with the desired characteristics and stability and to kill any potential spoilage or pathogenic microorganisms. Processing may be performed in batch cookers (discontinuous process) or in continuous cookers connected to supplies of water, steam and vacuum. Processed cheese in the Czech Republic are produced mainly in a discontinuous way using the temperature of 90 - 100 °C with the total melting time between 5 - 15 minutes. Higher temperatures 130 - 140 °C can be achieved in continuous cookers where the temperature is hold for 5 - 20 s and then cooled to 70 - 95 °C for 4 - 15 minutes to allow adequate interaction of the different blend ingredients and develop the desired textural characteristics [95, 106].

For most packaging formats, the processed blend is conveyed from the cooker to the filling machines, sometimes via an intermediate buffer tank with gentle agitation. Numerous packaging formats are available, e.g. individually wrapped portions (foil-wrapped triangles), blocks, tubes or slices. To prevent microbial contamination, the product should be packaged immediately after processing and the temperature should not fall below 60 - 70 °C [107]. Packaged and cooled processed cheese should be stored in the temperature of 4 - 8 °C.

1.3.2. Microbiology of processed cheese products

Cheese processing normally involves the heat treatment of ingredients at a temperature of 80 - 100 °C which is held for 5 - 15 minutes. During this process the majority of vegetative forms of microorganisms are inactivated, including

bacteria of the family *Enterobacteriaceae.* Nevertheless, the temperatures used are not sufficient for killing bacterial endospores, although the spore-forming microorganisms are often weakened. 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 rod-shaped bacteria of the genus *Bacillus* and *Clostridium* [95, 96].
Bacteria may also gain access to processed cheese following manufacture; contamination during production, wrapping and distribution of these products is also possible. Processed cheese are prone to contamination by micromycetes, which can grow at cold storage temperatures in an environment with the atmosphere containing lower concentration of oxygen. In most cases, spoilage is caused by representatives of the genus *Penicillium*. Besides above mentioned microorganisms, other species have been isolated from processed cheese as a result of secondary contamination [95, 97].
Microbial quality of processed cheese products depends on the quality of ingredients, as well as on other factors such as pH value, moisture content, fat content or the presence of additives. The presence and growth of microorganisms in processed cheese products can be minimized by the addition of preservatives, such as nisin, ascorbic acid, sodium sorbate or sodium propionate. Another approach for safer product is carefully considered formulating to maintain the pH, water activity and moisture at a level not favourable for microbial growth [97, 108]. Bacterial spoilage can be effectively eliminated by keeping good manufacturing practice, minimization of manual handling of product, avoiding post-processing contamination and reducing storage temperature [109, 110].

1.3.2.1. Microbial contaminants of processed cheese products

As mentioned above, the contamination and spoilage of processed cheese products are often caused by spore-forming bacteria. Highly undesirable microorganisms of significance in processed cheese products include gram-positive genera *Bacillus* and *Clostridium.* While *Clostridium* sp. cells are obligate or aerotolerant anaerobic bacteria, *Bacillus* sp. are aerobes. These genera form gram-positive rods and produce spores which are especially resistant to elevated temperatures [111]. Indeed their subsequent germination is frequently triggered by exposure to such temperatures, and their common occurrence in the environment makes this a potent source of spoilage and food poisoning bacilli and clostridia. The heat resistance exhibited by the bacterial endospore is due to its ability to maintain a very low water content in the central DNA-containing protoplast. The relative dehydration of the protoplast is maintained by the spore cortex, a surrounding layer of peptidoglycan which is also responsible for the spore´s reflactile nature [112]. Although the heat

resistance has been the major concern of medical and food microbiologists, spores also have increased resistance to other stresses. These include deleterious chemicals, ultraviolet radiation, desiccation or freezing. Endospores remain viable even in the absence of nutrients for periods of time that are unequalled by other life forms [113-115].

Resistance to various chemical and physical agents appears at different stages during sporulation, concomitant with changes in the physico-chemical composition of the sporulating cell. The sequence of morphological changes during spore formation has been divided into series of stages. Formation of an axial filament of chromosome extending across the length of the bacterium is designated as stage I. This filament consists of two copies of chromosome, with a chromosome origin region located near each cell pole. Then, there is an asymmetrically located division resulting in the formation of two unequally sized cells, the larger being the mother cell and the smaller prespore, which will develop into the mature spore. Completion of this division is designated as stage II. In stage III, the prespore becomes entirely engulfed by the mother cell. Two types of cell wall material, the cortex and the primordial cell wall, are deposited between the opposed membranes that surround the prespore (stage IV) and several protein layers, known as spore coat, are then assembled on the surface of the prespore (stage V). During stage VI and VII the prespore matures into the resistant spore and the prespore cytoplasm becomes dehydrated. Finally the mother cell lyses, releasing the mature spore (stage VII) [113]. This dehydration is mainly caused by the massive synthesis of dipicolinic acid. Dipicolinic acid is a spore-specific component present in high concentration in all bacterial spores and along with calcium ions is required for heat resistance [115].

Endospores are capable of germination and outgrowth under the proper environmental conditions. Outgrowing spores commence and continue vegetative growth, characterized by exponentially increasing cell number, until some required component of the culture medium becomes limiting. When this occurs exponential growth ceases and sporogenesis commences. The ultimate product of sporulation process is a free spore with the potential for repeating the entire process [114].

Major microbial contaminants of processed cheese are briefly characterized below.

Genus *Clostridium*

The hallmark of members of the genus *Clostridium* is a combination of phenotypic characteristics, i.e. rod-shaped morphology, endospore formation, gram-positive staining behaviour and anaerobic, fermentative metabolism in which sulphate is not reduced dissimilatorily [116]. Most of the species form straight to slightly curved rods of different lengths and diameters, the ends of which vary from rounded and squared to tapered or pointed. Clostridia usually

form only one spore either round or oval which is terminally, subterminally or centrally located in the mother cell. Spores are formed either with or without distending or swelling of the mother cell [117].
The physiology and metabolism of members of the genus *Clostridium* vary notably and utilize many different metabolic principles. The spore-forming anaerobes of the genus *Clostridium* can be either predominantly proteolytic or saccharolytic but both activities are normally accompanied by gas production [112].
Distinctive types of infection have been associated with certain species of *Clostridium:* gastrointestinal illness with *Clostridium perfringens* and *Clostridium difficile*; neurologic syndromes with *Cl. botulinum* and *Cl. tetani*; focal suppurative infections, myonecrosis and gas gangrene with *Cl. perfringens, Cl. novyi, Cl. septicum* or *Cl. histolyticum*; and bacteremia with *Cl. perfringens* and *Cl. septicum* [118].
Clostridia associated with contamination of cheese, milk and processed cheese products are *Cl. butyricum, Cl. perfringens, Cl. botulinum, Cl. sporogenes, Cl. tyrobutyricum, Cl. difficile* and *Cl. cochlearium* [97, 119, 120].

Clostridium perfringens
Cl. perfringens is commonly found in human and animal faeces and is widespread in the environment in soil, dust and vegetation. The cells need several amino acids for growth; therefore they multiply very effectively in many protein foods. The organism is a common contaminant of meat and poultry and can also contaminate dairy products. It forms oval, central spores rarely seen in culture. The spores are readily formed in the intestine and enterotoxin is produced on sporulation in the gut. Foods contaminated with large number of vegetative cells of *Cl. perfringens* can give rise to illness characterized by diarrhoea and abdominal pain. Spores have been reported also in raw milk and cheese, but there have been few reports of illness associated with bacterium in milk [97, 121].

Clostridium difficile
Cl. difficile has been recognized as the causative agent of a broad spectrum of enteric disease ranging from mild antibiotic-associated diarrhoea to pseudomembranous colitis. The cells are 3-5 µm in length and stains predominantly gram-positive, although older colonies may exhibit marked Gram stain variability. Sporulation is most noticeable on agar cultures that have reached a stationary or decline phase after more than 72 h incubation. Oral ingestion of *Cl. difficile* spores leads to the colonization of the colon in some patients following antibiotic exposure, which reduces the ability of the normal flora to resist colonization. Diarrhoea and colitis is related to exotoxins produced by *Cl. difficile.* The presence of *Cl. difficile* in the colon does not always infer active infection, as asymptomatic carriage has been reported in up to 14% of

hospitalized adults receiving antibiotics, and up to 70% of healthy infants [122, 123].

Clostridium botulinum

The cells of *Clostridium botulinum* are motile with peritrichous flagella, obligatory anaerobic, straight or slightly curved rods 2 - 10 µm long, and form central or subterminal oval spores. Physiological diversity within species *Cl. botulinum* is recognized by its division into four groups and molecular studies based on DNA homology and rRNA sequences have confirmed this grouping. Group I strains are strongly proteolytic and their presence in food is betrayed by partial disintegration of the product and a slight rancid or cheesy odour. These strains are of little concern in adequately refrigerated products, because they are not psychrotrophic. They do, however, produce spores with the highest heat resistance, thus pose a problem in foods that are underprocessed in a heating step.

In contrast, Group II strains represent a greater potential hazard in chilled foods, as they can grow and produce toxins at even 3 °C. These strains are non-proteolytic a produce spores with a low resistance to heat [112].

The species *Cl. botulinum* is divided into seven toxigenic types, A through G, on the basis of the serological specificity of the neurotoxins they produce. Botulism is a neuroparalytic illness caused by neurotoxins produced by *Cl. botulinum*. Foodborne form of botulism is caused by ingestion of preformed toxin in contaminated foods, with home-canned or prepared foods being the most often implicated vehicle. On a weight basis, botulism toxins are the most potent poisons known. Following ingestion, toxin molecules are absorbed from the upper portion of the intestine through the intestinal wall and spread via blood to the peripheral nerves. Its toxicity relates to the ability of the toxin to cleave one or more proteins by which neuronal vesicles release acetylcholine into the neuromuscular junction. Thus, toxins block signal transfers, irreversibly causing paralysis of all involuntary muscles [118, 124].

Clostridium butyricum

The metabolism of *C. butyricum* is primarily saccharolytic. *Cl. butyricum* stop growing when exposed to O_2 but resume growth under reinstated anoxic conditions [117]. This bacterium is capable of growing in acidic environment at pH 4.0 and is often a cause of spoilage in canned acid foods [125]. Endospores are central to subterminal and oval in shape without distending the mother cell. Like a number of other mesophilic clostridia, *C. butyricum* has been recovered from wounds and abscesses, but it is not a pathogen in the same sense as *Cl. tetani.* The importance of *Cl. butyricum* to food hygiene has proved to be remarkable in many aspects. It is a spoilage bacterium capable of forming butyric acid in foods and certain strains carrying the gene for botulinal neurotoxin may cause foodborne intoxication. *Cl. butyricum* was considered a

non-pathogen clostridium until 1986, when two cases of botulism linked with this species were reported in Italy [126]. Neurotoxigenic *Cl. butyricum* type E has been implicated in infection botulism and has lately been shown to be an evolving foodborne pathogen [127].

Clostridium tyrobutyricum

Cl. tyrobutyricum often contaminates raw milk and diverse cheese varieties. Growth of this spore-forming organism during cheese ripening may result in defects such as off-flavours and late gas blowing. Late gas blowing in hard and semi-hard cheese varieties is caused by an anaerobic metabolism of lactate to butyrate and H_2 [95, 128]. This problem can be overcome by good hygiene, addition of $NaNO_3$ or lysozyme or by the physical removal of the spores by bactofugation or microfiltration. The principal sources of *Cl. butyricum* are soil or silage. Grass silage used as a feed in winter may represents the main source of contamination of milk with spores of *Cl. butyricum* as it contains large number of spores that survive passage through the digestive tract of cows [96].

Genus *Bacillus*

This genus contains rod-shaped aerobic or facultative anaerobic spore-forming bacteria. Cells are straight and vary widely in size, appear in chains or as single cells. Endospores may be located centrally, sub-terminally or terminally and their shape varies from spherical to elliptical. Representatives include psychrophilic, mesophilic and thermophilic species. The genus still remains very large, with over 150 species, as transfers of species to other genera such as *Geobacillus* or *Paenibacillus* have been balanced by proposals for new *Bacillus* species [129, 130].

Bacilli are widely distributed in the natural environment as saprophytes, and their most frequent habitats are soils of all kinds, ranging from alkaline through neutral to acid, and the water columns and bottom deposits of fresh and marine waters. Many of them degrade biopolymers, with versatilities varying according to species and have important role in biological cycling of carbon and nitrogen. With the exception of *Bacillus anthracis* bacilli are not highly pathogenic to mammals, although *Bacillus cereus* has been associated with a range of opportunistic infections. Several species are important in foods, because they can cause foodborne disease (*Bacillus cereus)* and food spoilage, especially in canned product (*B. coagulans, Geobacillus stearothermophilus*) [85, 124, 129, 131].

Bacillus cereus

Bacillus cereus is facultative anaerobic with large vegetative cells, typically 1.0 µm by 3.0 - 5.0 µm, often arranged in chains. It grows over a temperature range from 8 to 55 °C with optima around 30 - 35 °C and does not have any marked

tolerance for low pH (minimum 4.9) or water activity (minimum 0.95). Spores are centrally located, ellipsoidal in shape and do not cause swelling of the mother cell. The spores show a variable heat resistance; recorded D values at 95 °C in phosphate buffer range between 1 min up to 36 min [112]. *B. cereus* is widely distributed in the environment and can be isolated from soil, water and vegetation. This ubiquity cause that it is also a common component of the transient gut flora in humans. Low numbers of this bacterial species can be found in a number of food products, including fresh and processed [85]. *Bacillus cereus* has been implicated in the spoilage of pasteurized milk and cause a defect known as bitty. Bacilli produce the enzyme lecithinase, which hydrolyses phospholipids of the fat globule membrane, causing aggregation of fat globules that adhere to container surfaces. Production of rennin-like enzymes can cause sweet curdling of milk at a higher pH than that required for acid curdling [132].
The incidence of food borne gastroenteritis by *B. cereus* origin is relatively high in some European countries, whereas low number of cases has been reported in United States [133]. The strains produce at least two types of enterotoxins (emetic and enteric), each probably associated with specific types of symptoms. Symptoms of the diarrhoeal syndrome resemble those of *Clostridium perfringens* food poisoning. The onset of illness is about 8 - 16 h after consumption of contaminated food, lasts for 12 - 24 h, and is characterized by profuse watery diarrhoea and abdominal pain. The emetic syndrome resembles the staphylococcal food poisoning with shorter incubation period and nausea and vomiting that last for 6 - 24 h. Both syndromes are caused by distinct toxins, diarrhoeal form is associated with heat-labile enterotoxins whereas emetic syndrome is connected with heat-stable emetic toxin cereulide. Toxins are produced during growth of cells at the growth temperature range and retained in the cells. Only when cells are lysed are the toxins released [85, 112, 124].
Far less common than outbreaks featuring *B. cereus*, a number of other *Bacillus* species have been associated with foodborne illness. These involve very closely related species such as *B. subtilis, B. licheniformis* and *B. pumilis* [112].

Other microorganisms contaminating processed cheese products

Contaminants of processed cheese products include microorganisms which survive the melting temperature, but there are many other microorganisms entering processed cheese as a result of secondary contamination. Examples of such bacteria which can contaminate processed cheese or raw materials designed for processed cheese production are *Staphylococcus aureus, Listeria monocytogenes, Salmonella* sp., *Escherichia coli, Pseudomonas* sp. or lactobacilli [95, 97].

Staphylococcus aureus

The genus *Staphylococcus* includes over 30 species of which approximately 20 are of real and potential interest in foods. Many of them are able to produce enterotoxins which are not destroyed by pasteurization [97]. Staphylococcal gastroenteritis is caused by the ingestion of food that contains one or more of these enterotoxins. Although enterotoxin production is believed generally to be associated with *S. aureus* strains that produce coagulase and thermonuclease, many species of *Staphylococcus* that produce neither coagulase nor thermonuclease are known to produce enterotoxins.

S. aureus is often present on human skin and in nasal area, thus it is considered to be a potential post-processing contaminant. The two most important sources for food contamination are nasal carriers and individuals whose hands and arms are inflicted with boils and carbuncles, who are permitted to handle foods [85].

Staphylococci are typical of other gram-positive bacteria in having a requirement for certain organic compounds in their nutrition. Amino acids are required as nitrogen sources, and thiamine and nicotinic acid are required among the B vitamins. When grown anaerobically, they appear to require uracil [124]. Environmental conditions required for growth are temperature of 7 - 46 °C, pH 5.2 - 9.0 and a_w higher than 0.86. *S. aureus* is known to grow in higher NaCl levels (10 - 20%) but is generally not a good competitor under conditions that allow growth of other bacteria [97].

Listeria monocytogenes

Recently, *Listeria monocytogenes* outbreaks have attracted worldwide attention. It is a widespread environmental contaminant and has been detected in raw milk with a greater frequency during cold weather months. The listeriae are widely distributed in nature and can be found on decaying vegetation and in soils, animal faeces, sewage, silage, and water and their association with certain dairy products is also well known [130].

L. monocytogenes is quite tolerant to high levels of sodium chloride and relatively low pH and refrigeration temperature. Its psychrotrophic characteristic may result in its growth on foods at low temperature [134]. These gram-positive bacteria are present in cheese factories even when good sanitation and hygiene are practiced and frequently contaminate drains, surfaces and cooling systems [97].

They grow well in many common media such as brain heart infusion, trypticase soy, and tryptose broths. *Listeria* sp. resembles most enterococci in being able to hydrolyze esculin, and grow in the presence of 10% or 40% (w/v) bile and in about 10% NaCl, but unlike the enterococci, they do not grow in the presence of 0.02% sodium azide [85, 124].

When *L. monocytogenes* is contracted via the oral route, it apparently colonizes the intestinal tract. From the intestinal tract, the organism invades tissues, including the placenta in pregnant women, and enters the blood stream, from

which it reaches other susceptible body cells. As an intracellular pathogen, it must first enter susceptible cells, and then it must possess means of replicating within these cells [112].

Escherichia coli

E. coli is an almost universal inhabitant of the gut of humans and other warm-blooded animals, where it is predominant facultative anaerobe generally considered a harmless commensal. Its common occurrence in faeces, ready cultivability, generally non-pathogenic character, and survival characteristics in water led to adoption of *E. coli* as an indicator of faecal contamination and the possible presence of enteric pathogens such as *Salmonella* Typhi in water. This usage has been transferred to foods where greater circumspection is required in interpreting the significance of positive results [112, 124].

Enterohaemorrhagic strains of *E. coli* (EHEC) particularly associated with the serotype O157:H7 has been recognized as the cause of a number of outbreaks of haemorrhagic colitis and haemolytic uremic syndrome, where foods such as raw milk and ground must have been implicated [112].

1.3.2.2. Factors that affect microbial growth in processed cheese products

pH

The acidity or alkalinity of an environment has a profound effect on the activity and stability of macromolecules such as enzymes, thus the growth and metabolism of microorganisms is influenced by pH value. Each species has an optimum and a range of pH for growth. In general, bacteria are more fastidious and prefer to grow at a pH near neutrality (pH 6.0 - 8.0), although there are exceptions, particularly among those bacteria that produce quantities of acids as a result of their energy-yielding metabolism. Examples important in food microbiology are the lactobacilli and acetic acid bacteria with optima usually around 5.0 - 6.0. Yeasts are more tolerant to lower pH values and their pH range of growth is 2.0 to 8.5. Filamentous fungi have the widest range of acceptable pH ranging from 1.5 to 9.0. On the basis of pH, foods can be grouped as high-acid foods (pH below 4.6) and low-acid foods (pH 4.6 and above). Most fruits, fruit juices and fermented foods are high-acid and are more commonly contaminated by yeasts and filamentous fungi. Bacteria primarily cause spoilage of proteinaceous food such as dairy, meat, poultry and seafoods with a pH range of 5.0 to 6.5 [12, 124].

When the pH in a food is reduced below the lower limit for growth of a microbial species, the cells not only stop growing but also lose viability, the rate of which depends on the extent of pH reduction. Adverse pH affects at least two aspects of a respiring microbial cell: the functioning of its enzymes and the transport of nutrients into the cell [85, 124]. Enzymes are proteins and their secondary and tertiary structure is largely maintained due to charge interactions

within the constituent amino acids. Adverse pH causes changes in these interactions, which result in modification of shape and therefore the biological activity of enzyme. Microorganisms tend to maintain an internal cytoplasmic pH at 6.5 - 7.0. The internal pH is tightly regulated and drops by approximately 0.1 unit for each 1.0 unit change in the environmental pH. Reduction in internal pH ultimately destroys the proton gradient between the inside and the outside of the cells and dissipates proton motive force as well as the ability of the cells to generate energy. Finally, microorganisms differ in their sensitivity to different organic acids. Weak acids, especially those that have higher dissociation constant (pK) are more antibacterial and organic acids also differ in their lipophilic properties, which in turn regulate their ease in entering the cells. For example acetic and propionic acids are more lipophilic than lactic acid and have therefore better antimicrobial effectiveness [85, 124, 135].

Increasing the acidity of foods is one effective way of limiting microbial growth. Adding an acidulant to the food or enhancing natural fermentation to develop acidity change the pH and can also shorten sterilization times owing to the lowered resistance of microorganisms in foods with increased acidity. In addition, the continuous presence of acid can effectively inhibit germination and outgrowth of spores that survive the thermal process. However, these actions tend to be microbistatic rather than microbicidal. Success in limiting the numbers of microorganisms will depend on the species of microorganism, the type and concentration of the acidulant, time of exposure, the buffering capacity of the food, and any preexisting conditions in foods that could enhance inhibition [12].

The minimum pH required by proteolytic clostridia, under otherwise ideal conditions, is 4.6 - 4.8. Non-proteolytic clostridia are able to grow in pH of the environment higher than 5.0 [97]. Anniballi *et al.* [136] determined the temperatures and pH levels that were most conductive to the growth and toxin production by the six strains of neurotoxigenic *Cl. butyricum* implicated in outbreaks of foodborne botulism. The strains were cultured for 180 days at various pH (4.6 - 7.0) and at temperatures of 10, 12, 15 and 30 °C. The lowest pH at which growth and toxin production occurred was 4.8 and the lowest temperature was 12 °C. At pH 5.6 and 5.8, which is the common value for processed cheese products, the growth and toxin production in all strains was observed within 4 days of incubation.

Water activity

Water is a major factor in controlling both microbial growth and chemical reactions in food. The availability of water in the food rather than its amount is the limiting factor for microbial survival. The measure of available water, the water activity (a_w), is defined as the ratio of the water vapour pressure of food substrate to the vapour pressure of pure water at the same temperature [137].

Decrease in water availability leads to an increase in the lag phase of growth, i.e. a period of growth cycle in which cells adjust to their new environment and the growth rate is equal to the death rate. Low water activity causes also a decrease in growth rate and population density and the number of cells at stationary phase is growing [137]. This effect results from adverse influences of lowered water content on all metabolic activities as all chemical reactions of cells require an aqueous environment [85]. Microorganisms need water for transport of nutrients, nutrient metabolism, and removal of cellular wastes. They also retain a slightly lower a_w inside the cells than the external environment to maintain turgor pressure. If the free water in the environment is reduced, the free water from cells flows outside in an effort to establish equilibrium, which results in osmotic shock and plasmolysis [124]. Even a slight reduction in a_w can prevent its growth. A reduction in a_w from 0.955 to 0.950 in the environment reduces the intracellular water content by 50% in *Staphylococcus aureus* and reduces the cell volume by 44% in *Salmonella* Typhimurium [138].

Since the beginning, reduced a_w has been used throughout human civilization to preserve foods. Some of these include salted fish and meats, dried meat, vegetables and fruits, sweetened condensed milk, jams etc. The a_w of foods can be reduced by using one or more of three basic principles: removing water by dehydration, removing water by crystallization or adding solutes to bind water. The water activity is also influenced by other environmental parameters such as pH, temperature of growth, and oxidation-reduction potential. The range of a_w over which growth occurs is widest at the optimum temperature for growth and the presence of nutrients also increases the range of a_w at which the organisms can survive [85, 124].

Microorganisms differ in their requirements on water activity. Gram-positive bacteria are usually less sensitive to reduced a_w than gram-negative species. Gram-negative bacteria such as *Pseudomonas* sp. and most bacteria of the family *Enterobacteriaceae* grow only at a_w values above 0.96 and 0.93, respectively, whereas micrococci grow below a_w of 0.90 and staphylococci can even grow at a minimum a_w of about 0.86. The minimum a_w values for growth of filamentous fungi is 0.8, with xerophilic molds as low as 0.6. Most yeasts are able to growth at a_w 0.85 and osmophilic yeasts have the minimum a_w value of 0.6 [124].

Spore-forming bacteria do not grow below a_w of 0.93. The a_w need for spore-forming bacteria to sporulate and the spores to germinate is generally higher than the minimum a_w for their growth [124]. Spore germination and outgrowth of *Bacillus cereus* is prevented at a_w of 0.97 to 0.93 and the minimum a_w for *Clostridium perfringens* spore germination is between 0.97 and 0.95 [137]. Growth of proteolytic strains of *Clostridium botulinum* is inhibited by a_w values lower than 0.935. Nonproteolytic strains are more sensitive to low water activity and are inhibited by $a_w < 0.97$. These values correspond to 10% and 5% of salt in the aqueous phase, respectively. Salt levels used in processed cheese are

generally 5 - 8%, resulting in water activity range 0.91 - 0.96. These values are generally sufficient to prevent the growth of *Cl. botulinum* [97, 109].
Water activity can also affect the toxin production in processed cheese products. Tanaka *et al.* [139] observed no botulinal toxin production at a_w below 0.944, although slightly higher a_w value (0.957) supported toxin development. In a similar study, Eckner *et al.* [140] indicated that toxin can be produced in cheese spreads with a_w of 0.93.

Fat content

Lipids are, in general, less preferred substrates for the microbial synthesis of energy and cellular components. Many microorganisms can produce extracellular lipases, which can hydrolyse triacylglycerols. In many foods the action of these enzymes is associated with spoilage, whereas in other foods the enzymes are credited for desirable flavours [124]. Lipids may also provide a protected environment for microorganisms and can serve as a protective barrier against antimicrobials in the water phase of a product. Several studies verified that lipid material in media and high-fat foods decrease the antimicrobial activity of lipophilic antimicrobials, such as nisin, monolaurin (MAG C12:0) and sorbic acid [141, 142]. The mechanism of this interference has been proposed by McLay *et al.* [62] and Blaszyk and Holley [76]. Most of the microbial activity occurs in the aqueous phase of the food. Lipophilic antimicrobials may partition into the fat phase of the food, which reduces their effective biocidal concentration and thus their availability for antimicrobial action.
Ter Steeg *et al.* [109] in their study observed a delayed growth of anaerobic clostridia in reduced-fat processed cheese products compared with higher fat products having the same moisture, salt and pH. This conclusion is supported by Mehta and Tatini [143], who evaluated the microbiological safety of reduced-fat Cheddar-like cheese and concluded that cheese with lower fat content were less hospitable to many bacteria. Populations of *Listeria monocytogenes* and *Salmonella* sp. were inactivated more rapidly in a reduced-fat cheese produced with a carbohydrate-based fat-replacer than in full-fat cheese.
Lower fat content belongs to the factors that contribute to the botulinal safety of reduced-fat and fat-free processed cheese products. Several studies confirmed a delay in production of botulinal toxin by clostridia associated with fat reduction. When fat levels in processed cheese products were reduced from 15% to approximately 1%, production of toxin was significantly delayed. Botulinal toxin was detected 5 days after inoculation in processed cheese having 15% of fat whereas in samples with 1 % of fat toxin was not detected until 14 days after inoculation [142].
In addition to the intrinsic effect of fat level, reduced-fat and fat-free processed cheese products often include ingredients to enhance flavour or improve

functionality that is usually lost when eliminating full-fat cheese from the base. Such ingredients including fat replacers, sodium lactate or monolaurin (MAG C12:0) can also poses the ability to affect microbial growth and toxin production. Jaskari *et al.* [144] suggested that β-glucan-containing fat replacers can slightly stimulate the growth of several bacteria, including *Lactobacillus* sp., *Bifidobacterium* sp., *Bacteroides* sp. and *Clostridium* sp.
Soy-based flavour enhancers have also been reported to affect production of botulinal toxin in cheese. According to Glass and Johnsson [142] toxin production at 30 °C was inhibited for at least 6 weeks by the addition of 10% soy-based flavour enhancer.

Emulsifying agents

Emulsifying agents that are most commonly used for the production of processed cheese and processed cheese products are phosphate- or citrate-based salts. These salts, especially orthophosphate and polyphosphate emulsifiers can inhibit microbial growth. Many studies have demonstrated that the inhibitory activity of phosphates on bacteria is linked to the chelation of essential metal ions [119,145-147]. Lee *et al.* [147, 148] found that certain polyphosphates had a lytic effect on *Staphylococcus aureus* and concluded that the macromolecules bind to the cell wall, remain bound, and chelate structurally essential metals, which then destabilizes the cell wall and leads to lysis. In contrast, other investigators observed only a bacteriostatic effect on *S. aureus* and hypothesized that polyphosphates trigger leakage of magnesium from cells, loss of osmoregulation, and membrane damage [149].
In general, gram-positive bacteria appear to be more sensitive than gram-negative bacteria [119, 150-152]. However, some studies have demonstrated that trisodium phosphate has an important antimicrobial effect on gram-negative bacteria contaminating poultry meat [153, 154] and can be used as antimicrobial surface treatments to decrease populations of pathogens, prevent growth of spoilage microorganisms, and extend shelf-life of fresh poultry [155]. Numerous studies have been conducted to determine the antimicrobial properties of polyphosphates on *Salmonella* Typhimurium, *Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa* where polyphosphates manifested inhibitory effects on bacterial growth at concentrations commonly used in the food industry (0.2 - 0.8%) [148, 156].
Several studies confirmed the activity of polyphosphates on spore-forming bacteria. Maier *et al.* [157] described the influence of polyphosphates on *Bacillus cereus* cells. Phosphate concentration of 0.1% or higher had a bactericidal effect, which was dependent on the growth phase. The most rapid decreases in number of viable cells were observed in exponentially growing cultures where the cell counts decreased by approximately 3 log units within 6 to 8 h. In contrast, stationary-phase cells were not significantly affected.

Inhibitory effects of polyphosphates on growth, sporulation and spore outgrowth of another spore-forming bacterium *Clostridium perfringens* were examined by Akhtar *et al.* [158]. Sub-lethal concentrations of polyphosphates significantly inhibited sporulation of *C. perfringens* by reducing sporulating cells (heat-resistant cells) by 5–6 log units. Although *C. perfringens* spores were able to germinate in the presence of 1% sodium tripolyphosphate, their outgrowth was significantly inhibited. A reduction of survival of *C. perfringens* was observed also in meat samples treated with 1% sodium tripolyphosphate contaminated with spores of *C. perfringens.*

In addition to the activity in meat samples, long-chain polyphosphates have shown inhibitory effect on spore-forming and a range of other gram-positive organisms in processed cheese [119].

Phosphates have also been reported to suppress the growth of yeast genera frequently linked to food spoilage such as *Candida, Geotrichum, Trichosporon, Brettanomyces* and *Cryptococcus.* In their study, Suaréz *et al.* [150] observed yeast inhibition, at neutral pH, when polyphosphates with long chain at concentrations from 0.3 to 1.5% were used.

2. AIMS OF THE THESIS

The fundamental aim of the thesis was to evaluate monoacylglycerols as potential antimicrobial agents apllicable in the food industry, with a focus on monoacylglycerols of non-traditional acids. This aim can be divided into four sections, with each section devoted to a different nature of experimental work:

I. Preparation of monoacylglycerols

II. Purification of crude recation products

III. Evaluation of antimicrobial activity of monoacylglycerols *in vitro*

IV. Evaluation of antimicrobial activity of monoacylglycerols in food matrices

Within these sections the following objectives were suggested:

- preparation of monoacylglycerols of fatty acids differing in their structure including unsaturated fatty acids and fatty acids with odd number of carbon atoms
- preparation of monoacylglycerols of non-traditional acids (perfluoroundecanoic acid and adamantane-1-carboxylic acid)
- purification and characterization of reaction products
- selection of microbial targets for *in vitro* tests
- *in vitro* evaluation of antimicrobial activity of prepared MAGs on gram-positive and gram-negative bacteria, isolated endospores and filamentous fungi
- monitoring of changes in growth characteristics of gram-positive and gram-negative bacteria caused by MAGs
- selection of MAGs with the widest range of antimicrobial activity
- production of processed cheese samples inoculated by spore-forming bacteria
- evaluation of inhibitory activity of MAGs on spore-forming bacteria in processed cheese samples

3. METHODS

The experimental part of the thesis comprises of four parts. The first phase focused on preparation of monoacylglycerols and included the optimization of reaction conditions, purification of reaction products and characterization of prepared monoacylglycerols. The optimization of preparation process was performed with the intention of achieving products with the highest possible reaction conversion. In the second phase of experiments, crude reaction products were purified in order to decrease the levels of unreacted components and to remove residual catalyst ions. The purity of prepared monoacylglycerols was evaluated by chromatographic methods and special attention was paid to monoacylglycerol of adamantane-1-carboxylic acid, a non-traditional monoacylglycerol that had not been synthesized yet. The third phase was aimed to define and evaluate effects of purified MAGs on bacteria, isolated endospores and filamentous fungi *in vitro.* The evaluation of antibacterial activity of selected MAGs in real environment of processed cheese was established as the main goal of the thesis and this analysis was carried out in the last phase of experimental part.

3.1. PHASE I. PREPARATION OF 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. The reaction was performed under fixed conditions of pressure and temperature in an open glass reactor equipped with a magnetic stirrer. A temperature-stabilized jacket of the reactor was capable of maintaining the reaction temperature within ± 0.5 °C of that required for the reaction. Briefly, the procedure consisted of several steps: melting the acid in a preheated reactor, addition of an appropriate amount of catalyst and subsequently adding glycidol. Taking into account that solubility of starting acid and catalyst is an important factor that affects reaction course, two options of reaction differing in their reaction medium, had to be considered. Concerning fatty acids, such as caprylic, capric or lauric acid, possess a low melting point, the reaction can readily proceed in the absence of solvent. Possessing a melting point as high as 174 °C, the reaction with adamantane-1-carboxylic acid had to be conducted in a suitable solvent. The solvent quantity affects the reaction process and reaction yield as well as other factors such as reaction temperature, reactant ratio and the amount of catalyst. All these parameters were examined and optimized individually for the particular acid by examining the reaction conversion rate.
The following monoacylglycerols were prepared:

MAG of caprylic acid (MAG C8:0, monocaprylin)
MAG of capric acid (MAG C10:0, monocaprin)

MAG of lauric acid (MAG C12:0, monolaurin)
MAG of undecanoic acid (MAG C11:0)
MAG of perfluoroundecanoic acid (MAG PFUNDA)
MAG of undecenoic acid (MAG C11:1)
MAG of oleic acid (MAG C18:1, monoolein)
MAG of adamantane-1-carboxylic acid (MAG ACA)

All prepared MAGs were examined for their content of unreacted acid by titration. At selected time intervals within the reaction process, samples were taken and examined by titration with 0.1 M ethanolic KOH to the phenolphtalein endpoint. The conversion of an acid was calculated from arithmetic means of three acid-base determination.

3.2. PHASE II. PURIFICATION AND CHARACTERIZATION OF MONOACYLGLYCEROLS

Crude reaction products were purified by filtration and recrystallization from ethanol. Crude MAG was dissolved in ethanol, heated, filtered through a Büchner funnel, cooled and placed in a freezer at -18 °C for 24 h. Formed crystals were subsequently separated by filtering, melted by heating and dissolved in ethanol. Recrystallization was repeated until pure crystalls of white colour were obtained. Following purification, the conversion of the acid was determined by titration.
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. Monoacylglycerol of adamantane-1-carboxylic acid, as a newly prepared compound, was further analysed by gas chromatography-mass spectrometry (GC-MS) and thermal properties of this MAG were observed using differential scanning calorimetry (DSC).
HPLC analyses were conducted using Waters chromatograph (Waters 600E pump, USA) supplemented with a RI detector. A reverse phase Bondapak C18 125 Å column (3.9 x 300 mm) and an elution system consisted of acetonitrile, water and acetic acid (80:20:1) were used. Results evaluation was carried out using Windows-based software CSW Clarity.
GC-MS analyses were run on a Shimadzu QP-2010 instrument using Supelco Equity1 (30 m, 0.32 mm) column, He as carrier gas at constant linear flow mode (59 cm/s, 50 kPa); 40 °C/5 min, 25 °C/min to 250 °C, hold for the required time. Electron impact ionization was used, ion source temperature: 200 °C, 70 eV. Only peaks of relative abundance exceeding 5 % are listed.
NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at frequencies of 300.13 MHz (^{1}H) and 75.77 MHz (^{13}C). ^{1}H- and ^{13}C-NMR

chemical shifts were referenced to the signal of solvent (^{1}H: (residual $CHCl_3$) = 7.27 ppm; ^{13}C: ($CDCl_3$) = 77.23 ppm.
Thermal properties of adamantane-1-carboxylic acid and MAG ACA were tested using Differential Scanning Calorimetry (DSC 1 Pyris Perkin-Elmer). The samples for analyses weighed ca 15 mg were sealed in an aluminum pans. The temperature and heat flow of DSC signal were calibrated on heating at 10 °C/min using indium standard. Two kind of measurement were provided. The first was single heating from -30 °C to 250 °C at a heating rate of 10 °C/min after 1 min of specimens annealing at -30 °C. The second kind represents closer testing of 1-MAG derivative thermal behaviour in temperature range between -15 °C and 40 °C. The specimen was subject to selected temperature program representing cyclic annealing/cooling/annealing/heating procedure; annealing at 40 °C for 5 min, cooling to -15 °C cooling rate 10 °C/min, annealing for 1, 8 or 16 min, followed by heating to 40 °C by heating rate 10 °C/min. DSC data were recorded during specimen heating.

3.3. PHASE III. ANTIMICROBIAL ACTIVITY OF MONOACYLGLYCEROLS IN VITRO

3.3.1. Selection of microbial targets

Given that a main goal of the thesis is to find antimicrobial agents with potential application in food products, microorganisms contaminating these products were selected. Among bacteria there are two spore-forming species *Bacillus cereus* and *Bacillus subtilis.* Some bacilli strains can cause foodborne disease and food spoilage and they produce extracellular enzymes that hydrolyze food components. *Micrococcus* sp. and *Staphylococcus* sp. were selected because of their potential for common transfer by human hands, as both genera are found on human skin. *Micrococcus luteus* can cause food spoilage, *Staphylococcus aureus* strains are frequently involved in foodborne diseases and can produce toxins at ambient temperatures. *Enterococcus faecalis* is found in human and animal intenstine and serve as an indicator of sanitation. Enterococci are also important in food spoilage.
Pseudomonas sp. and *Serratia* sp. are often included in food spoilage due to the ability to metabolize wide variety of carbohydrates, proteins and lipids in food. *Escherichia coli* and *Citrobacter freundii* are commonly found in the intestinal contents of humans and animals. Both species are included in coliform group as an indicator of sanitation. *Salmonella enterica* is a major cause of foodborne diseases and was selected also because of its incidence in dairy products and its ability to growth at ambient temperature.

The inhibitory effects of selected monoacylglycerols were tested in the following strains of bacteria:

Gram-positive bacteria:
Bacillus cereus CCM 2010
Bacillus subtilis subsp. *spizizenii* CCM 4062
Enterococcus faecalis CCM 4224
Micrococcus luteus CCM 732
Staphylococcus aureus subsp. *aureus* CCM 3953

Gram-negative bacteria:
Citrobacter freundii CCM 7187
Escherichia coli CCM 3954
Pseudomonas aeruginosa CCM 3955
Salmonella enterica subsp. *enterica* ser. Enteritidis CCM 4420
Serratia marcescens CCM 303

All strains were obtained from the Czech Collection of Microorganisms (CCM, Czech Republic, www.sci.muni.cz/ccm/). Bacteria were grown on nutrient agar (Proteose beef extract 3 g/l, Proteose peptone 5 g/l, agar 15 g/l all supplied by Hi-Media, India, and NaCl 3 g/l supplied by Sigma-Aldrich, USA) and incubated at 37 °C or 30 °C.
Microbial suspensions for *in vitro* tests were prepared by inoculating 5 ml of nutrient broth (Proteose beef extract 3 g/l, Proteose peptone 5 g/l supplied by Hi-Media, India, and NaCl 3 g/l supplied by Sigma-Aldrich, USA) and grown at a temperature of 30 °C for 24 h.

The inhibitory effects of selected monoacylglycerols were tested in the following filamentous fungi:

Alternaria alternata
Aspergillus niger
Mucor racemosus
Penicillium roqueforti.

All strains of micromycetes were obtained from the collection of Department of Food Technology and Microbiology at Tomas Bata University in Zlin (Czech Republic). Filamentous fungi were aseptically sampled and incubated on Fungal agar (Hi-Media, India) at 25±1 °C for 7–14 days.

3.3.2. Observation of the effect of monoacylglycerols on bacterial growth in nutrient broth

Stock solutions of MAGs were prepared at a concentration of 20 g/l in absolute ethanol (LachNer, Czech Republic), sterilized by filtration (Millipore with the porosity of 0.22 µm) and stored in closed test tubes at a temperature of 4 °C.
Antimicrobial assays were performed through a microdilution method. Corresponding quantities of ethanolic stock solutions of MAGs were added to nutrient broth, so that the final concentrations of MAG were 25, 50, 100, 250, 500, 1000 and 1500 µg/ml. Before inoculation, the pH of the cultivation medium was adjusted to 6.8 - 7.0 using 1 mol/l NaOH or HCl. 200 µl volumes of each solution were dispensed into the wells of 96-well microtiter plate and 5 µl of the suspension of test organisms were added to each well. All tests were conducted in triplicate and controls were included. Wells filled solely with pure nutrient broth (i.e. without MAG) and inoculated with bacteria were used as a positive control. Non-inoculated wells containing nutrient broth with MAG at an appropriate concentration were used as a negative control.
Microtitre plates were incubated at 25 °C in a Microplate reader Benchmark (Bio-Rad, USA) for 24 h. The bacterial growth was observed spectrophotometricaly using the values of optical density (OD-values) of the cell suspension. The wavelenght for measuring OD-values was set at 655 nm with readings being taken every 30 min. Before each measurement, the sample was shaken for 10 s.
The values of optical density obtained from inhibitory assays were plotted against time to obtain the growth curves of the microorganisms tested and to enable analysis of growth inhibition caused by MAG. Results are expressed as the Growth index (G.I.) after 24 h calculated according to (1):

$$G.I = (OD_a/OD_c) * 100\ [\%] \qquad (1)$$

where: OD_c is the absorbance of the control and OD_a is the absorbance of the active sample at the time *t*.
The effect of a given MAG at a certain concentration on the microorganism tested was further evaluated according to the following growth indicators: lag-time (λ), maximal value reached (A) and maximum specific growth rate (μ_m). Since bacteria grow exponentially, it is often useful to plot the logarithm of the relative OD value against time (2):

$$y = \ln (OD_t/OD_0) \qquad (2)$$

where: OD_t is the optical density of the sample at time *t* and OD_0 is the absorbance of the sample at time 0.

The dependence of the logarithm of the relative OD value (y) on the time of cultivation (t) was described by means of the three-parameter Gompertz model (3):

$$y = A.\exp\left\{-\exp\left[\frac{\mu_m.e}{A}.(\lambda - t)+1\right]\right\} \quad (3)$$

where: μ_m is the maximum specific growth rate (h^{-1}) defined as the tangent in the inflection point; λ is the lag-time defined as the x-axis intercept of this tangent; and the asymptote A is the maximal value reached [$A = \ln (OD_t(OD_0)]$ [159].
The estimates of μ_m, λ and A parameters were determined by nonlinear regression analysis (nonlinear least squares regression) for the following conditions $\mu_m > 0$, $\lambda > 0$ and $A > 0$. The Marquardt-Levenburg method was applied by means of Unistat® 5.5 software (Unistat Ltd., UK). Quality of the designed models was evaluated by means of correlation coefficient (r). Correlation coefficients of modeled dependence of the logarithm of relative OD value on the time of cultivation were at an interval of 0.89 - 0.99.
Monoacylglycerols were added to culture media as ethanolic solutions due to low solubility in distilled water. To exclude the inhibitory activity of ethanol, experiments in which only ethanol was added to media were conducted. Similar test were also conducted with the Cr (III) catalyst used for the synthesis of MAGs. Neither chromium nor ethanol at the concentrations used in antibacterial assay had inhibitory effect on the growth of selected bacterial strains.

3.3.3. Observation of the effect of MAGs on isolated bacterial endospores in nutrient broth

Endospore suspensions were prepared by inoculating 10^5 - 10^6 cells into 10 ml of nutrient broth. Cultures of *Bacillus cereus* CCM 2010 and *Bacillus subtilis* subsp. *spizizenii* CCM 4062 were incubated at 30 °C on an orbital shaker (50 rpm). After incubation, the cell suspensions were gently poured onto the surface of nutrient agar plates (Proteose beef extract 3 g/l, Proteose peptone 5 g/l, agar 15 g/l all supplied by Hi-Media, India, and NaCl 3 g/l supplied by Sigma-Aldrich, USA). The plates were than incubated at 30 °C until sporulation. When free endospores were observed microscopically, between 1 and 2 weeks later, they were harvested from the plate surface by washing it five times with sterile distilled water. The washings were centrifuged at 10 000 rpm for 15 min at 4 °C and resuspended in 10 ml of sterile distilled water. Subsequently the suspensions were heated to 80 °C for 10 min and stored at -20 °C. Stored suspensions were checked for their viability and culture purity by streaking on nutrient agar plates [160].
Effect of MAGs on spore germination and outgrowth was observed in microtiter plates. Monoacylglycerols were added to nutrient broth to reach the final concentrations ranging from 25 to 1500 µg/ml. 250 µl of each solution were

dispensed into the wells of microtiter plate and inoculated with 5 µl of spore suspension. Microtiter plates were incubated at 30 °C for 7 days and every day the growth was observed spectophotometrically using Microplate Reader Benchmark. The values of OD at 655 nm were plotted against time to obtain growth curves. The micromethod described above was used to determine the MIC of the monoacylglycerols. The MIC was recorded as the lowest concentration of MAG that prevented the outgrowth of *Bacillus* spores and resulted in no visible growth throughout the incubation.

3.3.4. Observation of the effect of MAGs on the growth of filamentous fungi

The inhibitory effects of selected 1-monoacylglycerols were tested in the following filamentous fungi: *Alternaria alternata*, *Aspergillus niger*, *Mucor racemosus*, *Penicillium roqueforti*. Filamentous fungi were aseptically sampled and incubated on Fungal agar (Hi-Media) at 25±1 °C for 7–14 days.
Corresponding quantities of MAG solutions were added to individual doses of heat-sterilized Fungal Agar and dosed aseptically into Petri dishes. Growth was observed at the following concentrations of each MAG: 250, 750, 1000 and 1500 µg/ml. Micromycetes were inoculated into the agar using standard procedure. Fungal inoculum was taken by needle from the peripheral growth zone of stock cultures. Petri dishes with 20 ml of Fungal agar supplemented with MAG were inoculated by four times pinning. Samples were incubated for 14 days at 25±1 °C. Colony radii were measured after 7 and 14 days of incubation.
Each experiment was done in triplicate. The MIC values were determined from Petri dishes displaying no visible growth with the lowest MAG concentration.

3.4. PHASE IV. ANTIMICROBIAL ACTIVITY OF MONOACYLGLYCEROLS IN PROCESSED CHEESE

Processed cheese is produced by heating a mixture of natural cheeses with emulsifying salts under partial underpressure and constant stirring until a homogenous mass of desired properties is formed. During the melting process, the majority of contaminating microorganisms are inactivated, but spore-forming bacteria can survive at elevated temperature, thus provide a possible risk to the quality of the product and consumer health. Highly undesirable microorganisms in processed cheese belong to the group of rod-shaped endospore-forming bacteria of the genera *Bacillus* and *Clostridium.*
Survival of these microorganisms 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 survival. To evaluate antimicrobial activity of monoacylglycerols, processed cheese samples were treated with monoacylglycerols selected according to their antimicrobial activity *in vitro.*

3.4.1 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 was carried out. Ingredients and their levels are listed in Table 2. 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 minute. In the next step, samples were homogenized, packaged and cooled.
Except for ingredients listed in Table 2, monoacylglycerols were added 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 sensoric properties of processed cheese samples.
Before storage, the samples were inoculated with 5 ml suspensions of particular microorganism:

Bacillus cereus CCM 2010
Bacillus subtilis subsp. *spizizenii* CCM 4062
Clostridium butyricum CAPM 6342
Clostridium sporogenes CAPM 6329

Clostridia were obtained from Collection of animal pathogenic microorganisms CAPM), Veterinary Research Institute in Brno.

Table 2. *Composition of ingreients 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. $2(H_2O)$	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

3.4.2. Microbiological analysis of processed cheese samples

During storage, processed cheese samples were removed from incubation at the selected time intervals and microbiologically analyzed. In each case, 1 g of sample was aseptically removed, diluted 1:9 (wt/vol) 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 number of colony forming units per one gram of the sample (CFU/g) was calculated. Aerobic population levels were determined using Plate Count Agar (PCA, Hi-Media) with incubation at 37 °C for 48 h. Anaerobic plate counts for clostridia were performed with Reinforced Clostridial Agar (RCA, Hi-Media) under anaerobic conditions (7% CO_2) with incubation at 37 °C for 48 h.

4. RESULTS AND DISCUSSION

4.1. PREPARATION OF MONOACYLGLYCEROLS

Monoacylglycerols were prepared by Cr (III) catalyzed addition of particular acid to glycidol according to the procedure described in chapter 3.1. This reaction is one of the possible methods which lead to pure *sn*-1 monoacylglycerols. The regioselectivity of epoxide ring opening depends on several reaction conditions as well as on the type of catalyst used. Several studies conducted to date confirmed the effectiveness of the application of chromium (III) compounds as catalysts for preparing 1-monoacylglycerols of high purity, especially chromium (III) acetate is highly regioselective [34, 35, 40, 41]. The reaction mechanism is based on the formation of coordination bond between chromium (III) ion and the oxygen atom of the epoxide ring, folowed by acetate ion attack on the less substituted carbon atom of epoxide [40]. The quantity of Cr (III) catalyst affects the course of the reaction and the final fatty acid conversion. According to Janiš *et al.* [34], with an increasing amount of catalyst, the reaction time gets considerably shorter; on the other hand a greater contamination of the product by Cr (III) occurs. For all monoacylglycerols prepared for the experimental part, the dose of catalyst 0.5% by mass was considered to be optimal taking into account both aspects - the conversion and acceptable contamination of the products by Cr (III) ions.

Apart from catalyst type and quantity, there are other factors that affect the reaction process and quality of the product. The conversion of the acid is naturally affected by the reactant ratio [34]. Since the reaction mechanism represents a direct addition of the acid to glycidol, the reactant ratio can be set as equimolar (1:1). However, molar excess of glycidol usually results in a steep fall of the content of residual unreacted acid. Since the removal of unreacted glycidol from the reaction product is substantially easier than the removal of the residual acid, preparation of MAGs was conducted with a molar excess of glycidol.

The degree of conversion is affected by temperature, due to the fact that both the catalyst and the acid have to be completely dissolved. As it has been published earlier by Janiš et. al [34, 35, 41], a complete dissolution occurred when the temperature of about 90 °C were used. All monoacylglycerols were prepared at 90 - 96 °C. For monoacylglycerols of acids with high melting point, the reaction had to be conducted in a suitable solvent and the volume of solvent also affects the course of the reaction.

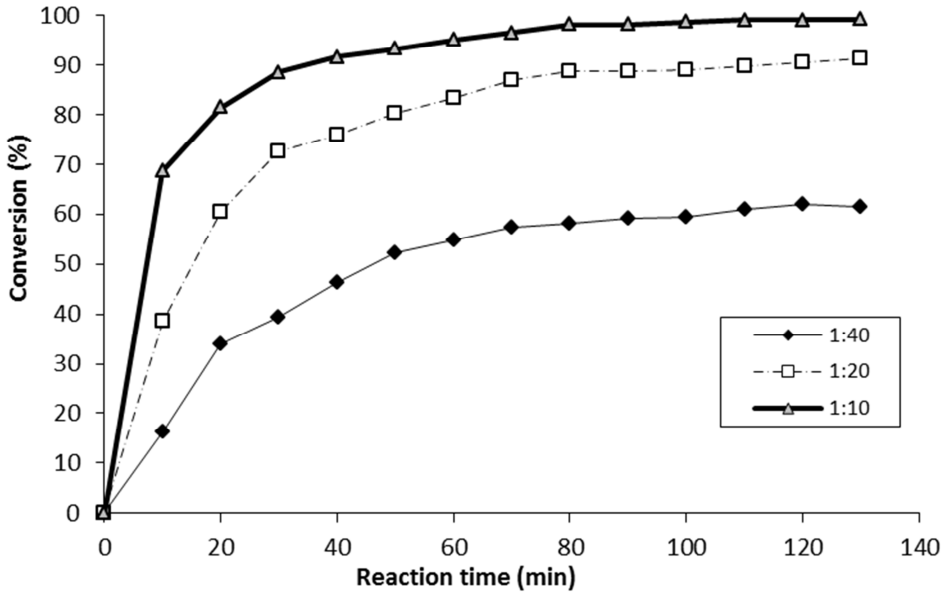
All above mentioned reaction parameters were optimized individually for each monoacylglycerol prepared. As an example of such optimization, the preparation of MAG of adamantane-1-carboxylic acid (MAG ACA) is discussed in more detail in the following paragraphs.

Preparation of MAG ACA

For the reaction of glycidol with adamantane-1-carboxylic acid, the temperature was finally set at 96 °C, further increases did not lead neither to acceleration of the reaction nor to higher conversion.

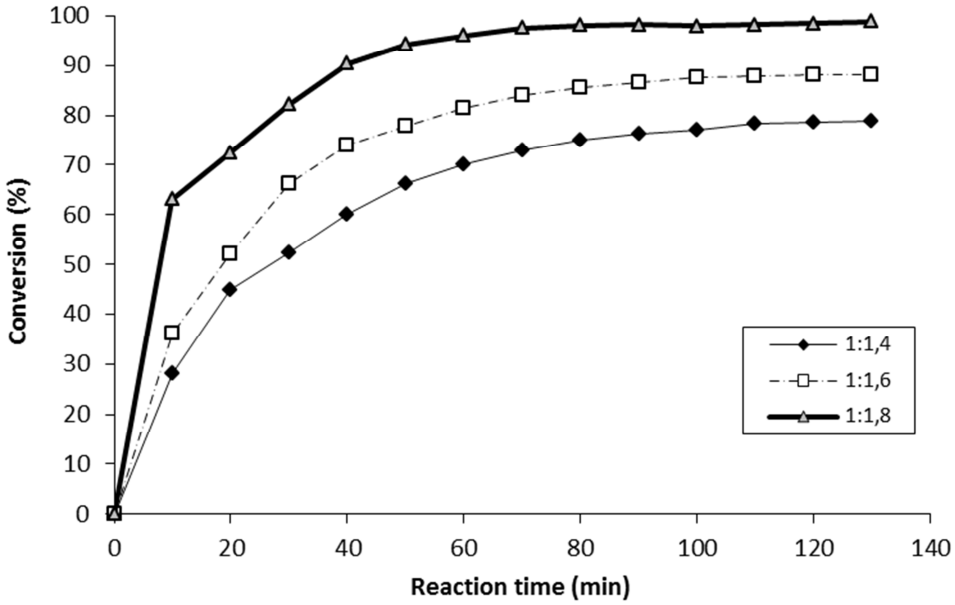
Possessing a melting point as high as 174 °C, the reaction with adamantane −1− carboxylic acid had to be conducted in a suitable solvent. The first group of tests was conducted in order to evaluate the effect of the solvent quantity on the reaction conversion rate. Three series of tests differing in the molar ratio of the acid and toluene as a solvent were conducted (Figure 1). With the molar ratio 1:40, the conversion did not exceed the 60% in 90 min, even a prolongation of the reaction time up to 150 min failed to further increase. With a decreasing toluene amount, the conversion rate was positively affected with 91.20±0.21% for the 1:20 and 99.13±0.24% for the 1:10 molar ratio. The usage of higher molar ratio did not allow the acid to dissolve completely and the conversion rates were significantly lower.

Figure 1. *Effect of the adamantane-1-carboxylic acid/toluene molar ratio on the conversion rate. Raction temperature 96 °C, glycidol/adamantane-1-carboxylic acid ratio 1:1.8, catalyst* $[Cr_3(CH_3COO)_7](OH)_2$ *0.5% by mass*



Another factor, that influenced the course of reaction and the purity of final reaction product, is the reactant ratio. With the reactant ratio set as equimolar (1:1), the conversion ranged from 40 to 50% (data not shown). Further tests were conducted with a molar excess of glycidol. The residual acid content decreased significantly and with the acid-glycidol molar ratio set at 1:1.8 the conversion rate achieved 98.93±0.39% (Fig. 2).

Figure 2. *Effect of adamantane-1-carboxylic acid/glycidol molar ratio on the conversion rate. Reaction temperature 96 °C, adamantane-1-carboxylic acid/toluene ratio 1:10, catalyst* $[Cr_3(CH_3COO)_7](OH)_2$ *0.5% by mass*



Apart from monoacylglycerol of adamantane-1-carboxylic acid, there is another MAG prepared from non-traditional acid, MAG of perfluorinated derivative of undecanoic acid (MAG PFUNDA). Similarly to MAG ACA, reaction of perfluoroundecanoic acid and glycidol was conducted in the presence of toluene as a solvent. Conversion of the acid reached the highest value (92%) under the following reaction parameters: perfluoroundecanoic acid/glycidol molar ratio 1:1,6; perfluoroundecanoic acid/toluene molar ratio 1:5; catalyst 0.5% by mass and the reaction temperature 96 °C.

As far as monoacylglycerols of traditional fatty acids are concerned, optimization of the reaction process yielded 95 - 99 % conversions of particular acids in all reaction products. Crude monoacylglycerols were subsequently purified and analysed by chromatographic methods.

4.2. PURIFICATION AND CHARACTERIZATION OF MONOACYLGLYCEROLS

Monoacylglycerols prepared by the addition of a particular acid to glycidol were purified using triple recrystallization from ethanol. In order to assess the Cr (III) ions content, both the crude and the purified product were analyzed by atomic absorption spectroscopy (AAS). Purification led to removing of residual glycidol and reducing the quantity of Cr (III) ions from 1500 µg/g in the crude reaction product to 500 µg/g in the purified product.

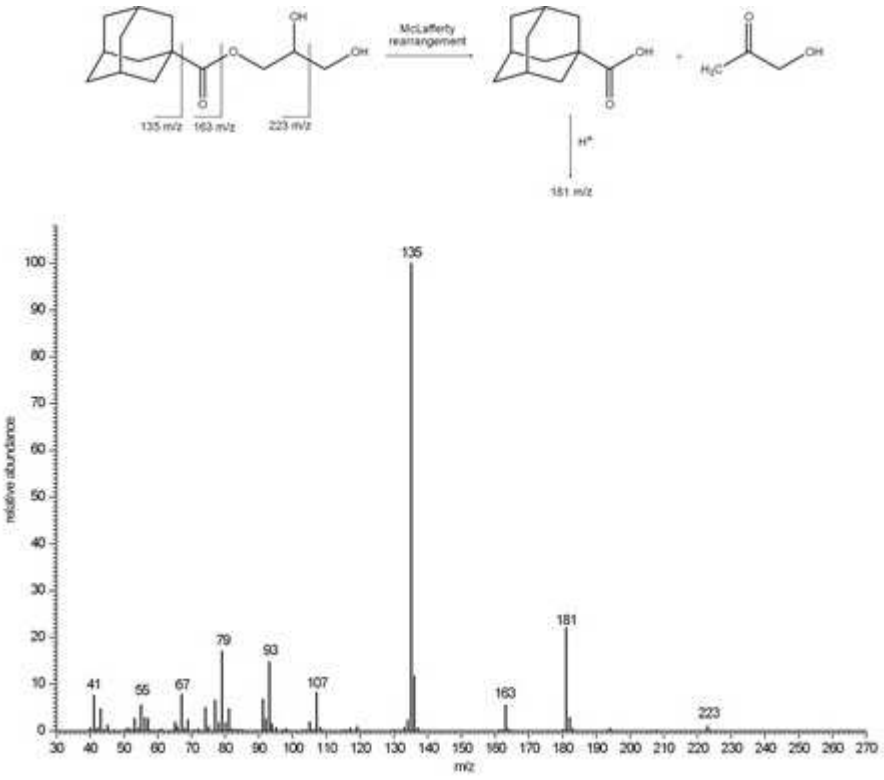
After the recrystallization of crude reaction products from ethanol, the purity of the obtained products was very high. Purified monoacylglycerols were analyzed

by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) and these analyses did not prove either significant formation of side products or reactant residues in MAG C10:0, MAG C11:0, MAG C11:1, MAG C12:0 and MAG C18:1. As for MAGs of non-traditinal acids (MAG PFUNDA, MAG ACA), residual acid, glycidol or *sn*-2 isomers of MAGs were detected by HPLC, but content of these impurities was not substantial.

GC-MS analysis of MAG ACA

Monoacylglycerol of adamantane-1-carboxylic acid as a new compound was further characterized by gas chromatography – mass spectroscopy GC–MS and NMR analysis. Upon the GC chromatogram may be stated, that single regioisomer of MAG was detected. Under analysis conditions described in experimental part, the retention time of MAG was 16.0 min. The mass spectrum of this compound and suggestion of fragmentation pathway of probable product are depicted in Figure 3.

Figure 3. *EI-MS spectrum and supposed fragmentation pathway of 2,3-dihydroxypropyl adamantane-1-carboxylate (MAG-ACA)*



The base peak at 135 m/z may be reasonably attributed to adamantyl cation radical, which commonly occurs in spectra of adamantane derivatives. The McLafferty rearrangement and consecutive proton attraction led to formation of protonated form of adamantane-1-carboxylic acid yielding the peak at 181 m/z. The observation of corresponding signal at 201 m/z in the spectrum of lauryl-MAG may support this suggestion. The glycerol residuum may by reasonable source of protons seeing that no similar signals were observed when trimethylsilylated MAGs were analyzed [161, 162].
Peaks with relatively high abundance at 107, 93, 79, 67, and 55 m/z originate from typical adamantane cage fragmentation. Molecular peak was not observed.

NMR analysis of MAG ACA

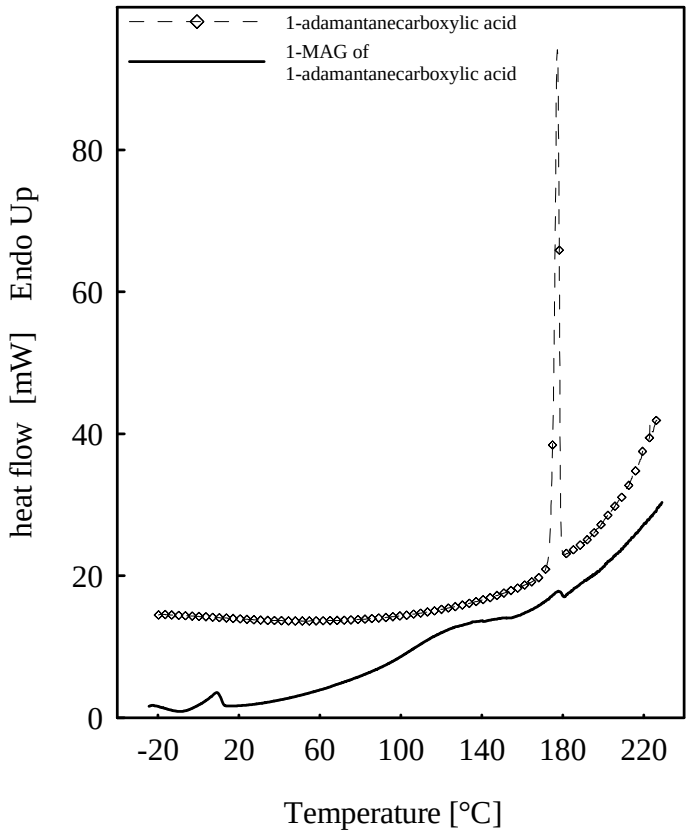
Spectral characterization of MAG ACA (2,3-dihydroxypropyl adamantane-1-carboxylate)
^{1}H NMR ($CDCl_3$): δ = 1.73 (m, 6H, CH_2(Ad)), 1.90 (m, 6H, CH_2(Ad)), 2.03 (m, 3H, CH(Ad)), 3.55–3.81 (m, 2H), 3.88–3.95 (m, 1H), 4.17 (m, 2H, $COOCH_2$) ppm. ^{13}C NMR ($CDCl_3$): δ = 28.1(CH), 36.6(CH_2), 39.1(CH_2), 41.1(C), 63.6(CH_2), 65.1(CH_2), 70.6(CH), 178.4(CO) ppm. EI-MS: 41(8), 43(5), 55(6), 67(8), 74(5), 77(7), 79(17), 81(5), 91(7), 93(15), 107(8), 135(100), 136(12), 163(6), 181(22) m/z (%).
The ^{1}H and ^{13}C NMR spectra of deuterochloroform solution of prepared compound proved the structure of asymmetrically substituted glycerol derivative. From two eventual regioisomers of MAG, only required 2,3–dihydroxypropyl adamantane-1-carboxylate matches this observation.

DSC analysis of MAG ACA

Thermal properties of adamantane-1-carboxylic acid and its 1-MAG derivative were tested using Differential Scanning Calorimetry. Calorimetric analyses of both principal materials are presented in Figure 4. Single thermal event can be clearly identified on DSC traces for adamantane-1-carboxylic acid. The observed sharp peak represents melting of adamantane-1-carboxylic acid's crystalline structure with temperature of melting evaluated as the maximum of peak 177.4 °C and heat of fusion representing the peak area calculated by peak integration to be 87.4 J/g.
The data for MAG-ACA are quite different when three thermal events were detected. There is only fractional response at the temperature range of adamantane-1-carboxylic acid melting. The peak maximum was detected at 177.8 °C with heat of fusion of 1.3 J/g. The origin of the presented peak is probably due to residue of adamantane-1-carboxylic acid when MAG conversion reaches some 98%. The second peak can be observed in temperature range 79.1-157.0 °C with maximum at 125.9 °C and endothermic enthalpy

change reaching 16.5 J/g. The origin of the second peak is probably due to additional evaporation of the toluene which is used as a solvent during MAG ACA synthesis. The third peak detected between -7.7 °C and -13.4 °C with maximum at 8.9 °C and enthalpy of transition to be 4.2 J/g is considered as a thermal response of prepared MAG ACA. Such relatively low value of transition together with fact that prepared matter is transparent indicates that MAG derivative represent amorphous solid mater. Accordingly, the third peak represents glass transition transformation of MAG ACA characterized by glass transition temperature, T_g. Enthalpy structural relaxation tests (physical aging) were further performed to support this idea [163, 164].

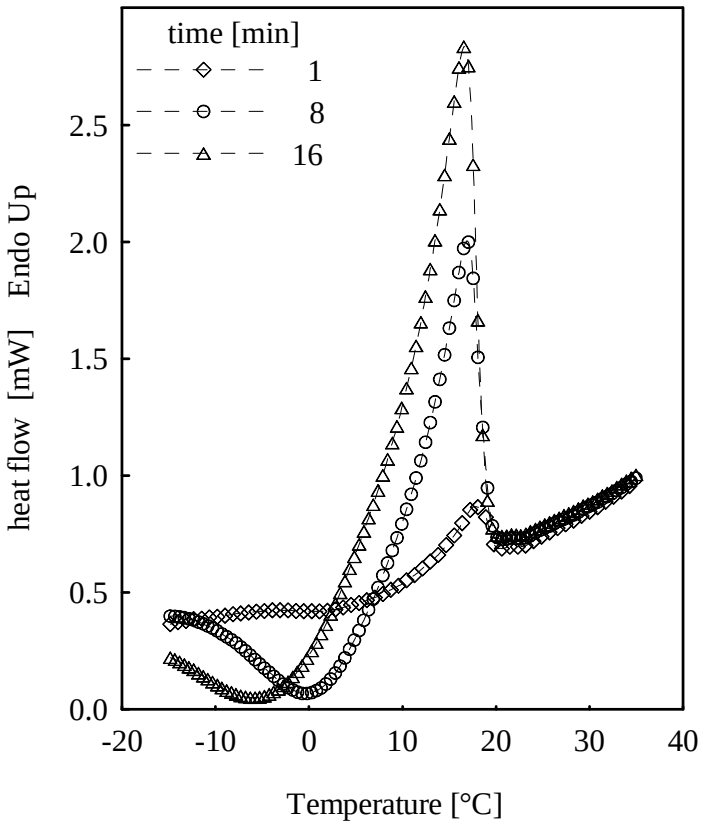
Figure 4. *DSC traces of adamantane-1-carboxylic acid and 2,3-dihydroxypropyl adamantane-1-carboxylate (MAG-ACA) during heating at 10 °C/min.*



Non-crystalline materials are usually found not to be in thermodynamic equilibrium after their transformation from melt to solid state leading to structural relaxation at temperatures below T_g. In our experiment the specimens were annealed at -15 °C<T_g for different times (1, 8 and 16 min). Relaxation below T_g cause enlarging of transition peak measured during subsequent heating. This relaxation phenomenon is clearly demonstrated by data presented in Figure 5. As was found the values of enthalpy needed for transformation from

glass to melt increase with time of annealing at -15 °C with values 1.3 J/g after 1 min, 2.7 J/g after 2 min and 4.9 J/g after 16 min of annealing at -15 °C.

Figure 5. *DSC traces of adamantane-1-carboxylic acid and 2,3-dihydroxypropyl adamantane-1-carboxylate (MAG ACA)*



4.3. EFFECT OF MONOACYLGLYCEROLS ON THE GROWTH OF GRAM-POSITIVE BACTERIA

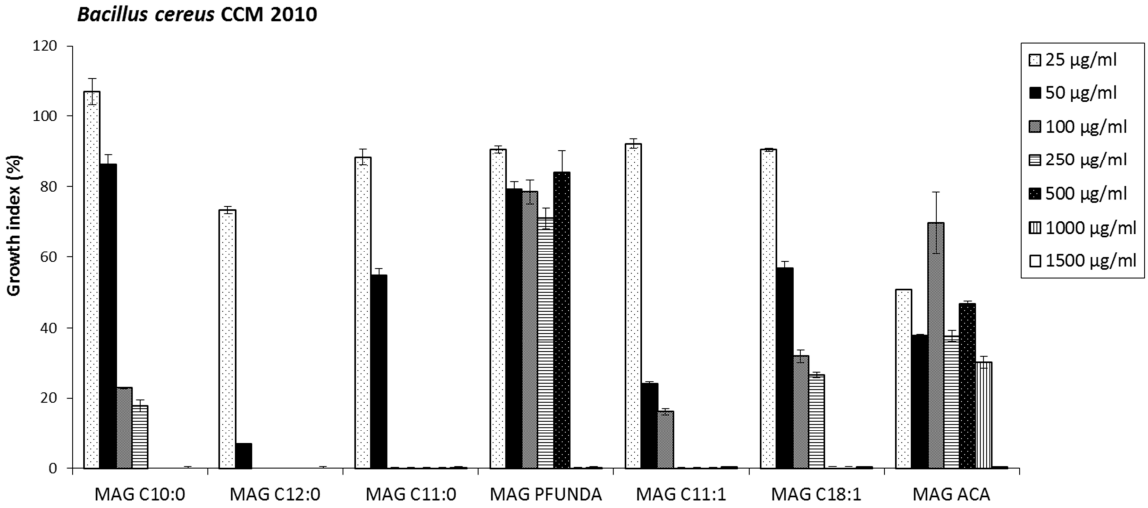
Seven monoacylglycerols differing in the structure of acid bound have been selected for testing their potential antimicrobial activity. Each monoacylglycerol falls into one of four categories. There are MAGs of saturated fatty acids with even number of carbon atoms (MAG of capric acid MAG C10:0, MAG of lauric acid MAG C12:0), MAGs of saturated fatty acids with odd number of carbon atoms (MAG of undecanoic acid MAG C11:0), MAGs of unsaturated fatty acids (MAG of undecenoic acid MAG C11:1, MAG of oleic acid MAG C18:1) and MAGs of non-traditional acids (MAG of perfluoroundecanoic acid MAG PFUNDA, MAG of adamantine-1-carboxylic acid MAG ACA).
Activity of these MAGs was examined on five gram-positive species cultivated in nutrient broth supplemented with MAGs at concentration 25 – 1500 µg/ml. Growth was observed by measuring OD values of bacterial suspensions for 24 h. Results are expressed as growth index which compares OD values of bacteria growing in nutrient broth supplemented with particular concentration of MAG with OD values of positive control, i.e. nutrient broth without MAG and inoculated by the corresponding microorganism. Data on the effect of MAGs on gram-positive strains are graphically presented and discussed in the following section.

4.3.1. *Bacillus cereus* CCM 2010

According to a literature survey *Bacillus* sp. vegetative cells seem to be relatively sensitive to the action of monoacylglycerols, especially monolaurin (MAG C12:0).
Mansour *et al.* [165] observed an inhibitory action of MAG C12:0 (250 µg/ml) on *B. cereus*, however monolaurin exerted bacteriostatic rather than bactericidal effect. Buňková *et al.* [49] found monolaurin (MAG C12:0) effective in reducing the number of *B. cereus* cells even at ten times lower concentration. Results obtained by measuring optical density are consistent with these observations, as monolaurin was the most effective in killing *B. cereus* of all monoacylglycerols tested (Figure 6).
A significant inhibitory effect on the growth of *B. cereus* was observed also after application of MAGs of fatty acids with odd number of carbon atoms. The total growth inhibition was detected in the presence of MAG of undecanoic acid (MAG C11:0) and MAG of undecenoic acid (MAG C11:1) at 100 µg/ml and 250 µg/ml, respectively.
Rather surprising were results of antibacterial activity of monocaprin (MAG C10:0). Monocaprin concentration ranges from 50 to 250 µg/ml are reported to cause a complete inhibition of *Bacillus* sp. [49, 65]. In this study, MAG C10:0 at 100 and 250 µg/ml reduced the growth index to approximately 20%, but complete inhibition was not achieved.

Although the addition of non-traditional monoacylglycerols (MAG PFUNDA, MAG ACA) led to a decrease in bacterial growth, a total inhibition was not recorded until higher concentrations (1000 – 1500 µg/ml) were used.

Figure 6. *Growth of Bacillus cereus CCM 2010 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



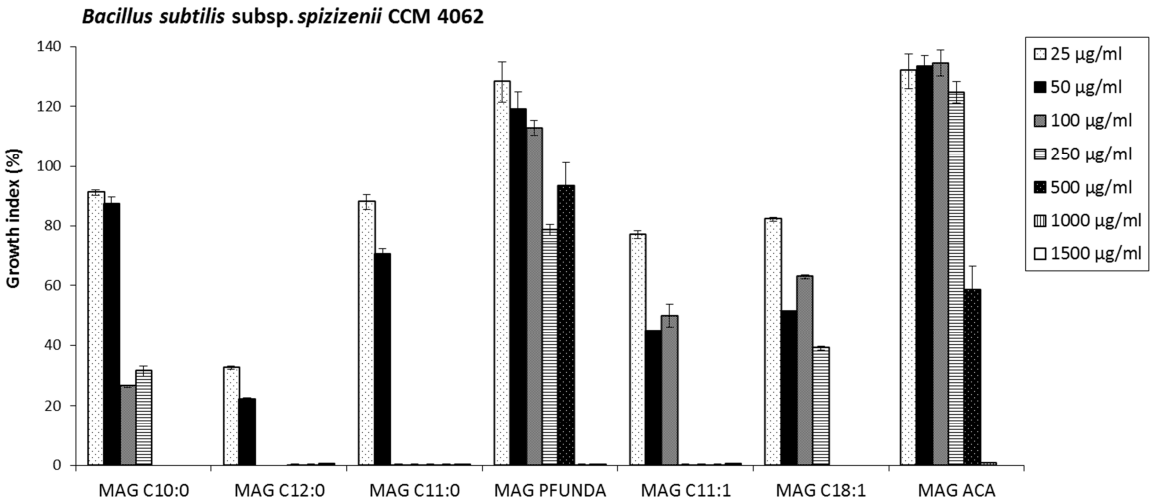
4.3.2. *Bacillus subtilis* subsp. *spizizenii* CCM 4062

Another species of the genus *Bacillus* was included in the group of microorganisms selected for the experimental part of the thesis. In a similar way to the manner in which monoacylglycerols inhibit *B. cereus, B. subtilis* is also significantly affected in growth by these compounds. Monolaurin (MAG C12:0) and MAGs with odd number of carbons in fatty acid chain appear to be the most efficient (Figure 7). These three MAGs caused a complete growth inhibition at concentration of 50 - 100 µg/ml. After the application of MAG C12:0 at the lowest concentration tested (25 µg/ml), the growth index decreased by 70%. This substantial antibacterial activity of monolaurin is in accordance with other authors, who have reported minimal inhibitory concentrations for bacilli within the range of 25 - 75 µg/ml [64, 166].

Monoacylglycerol of undecanoic acid (MAG C11:0) was the second most efficient within monoacylglycerols tested. In the presence of 25 - 50 µg/ml a slight inhibition occurred with growth index values 88.07 ± 2.51 and 70.45 ± 1.65, respectively. No visible growth was detected after addition of 100 µg/ml of MAG C11:0. Despite obvious efficacy of monoacylglycerol of undecanoic acid, its perfluorinated derivative MAG of perfluoroundecanoic acid (MAG PFUNDA) was active solely at high concentrations (1000 - 1500 µg/ml). The lowest concentration tested (25 µg/ml) exerted rather a stimulatory effect on growth of *B. subtilis* with the growth index 128.22 ± 6.8. These results suggest that the effectiveness of MAG against bacteria could be considered dose dependent and the same compound could exert an inhibitory or stimulatory

activity, because of its concentration. Similar phenomenon was reported for lauric acid by Altieri *et al.* [63] and could be explained by an increase in permeability of cytoplasmic membrane and faster exchange of nutrients between the interior and the environment.

Figure 7. *Growth of Bacillus subtilis subsp. spizizenii CCM 4062 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



4.3.3. *Enterococcus faecalis* CCM 4224

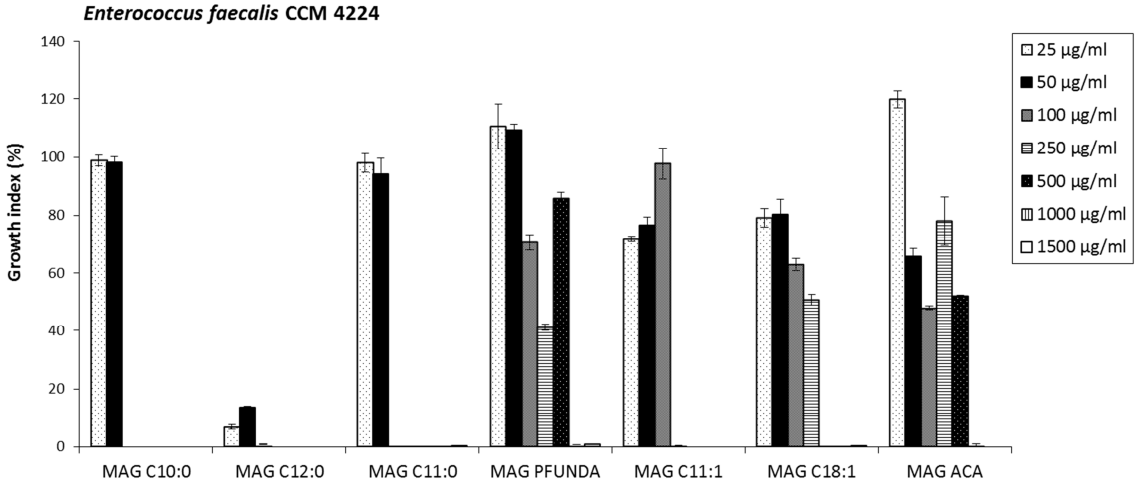
Enterococci are known to possess significant resistance to various adverse factors of environment. Since these gram-positive bacteria are able to survive some types of food processing, they have been implicated in outbreaks of food-borne illnesses and in the spoilage of processed cooked meat, raw meat, milk, and milk products [73].

There is an increasing concern about the emergence of drug resistant strains of enterococci. Dufour *et al.* [167] have reported isolation of monolaurin-resistant *E. faecalis* mutants. Authors observed differences in cell surface hydrophobicity between the wild type and the mutant, with the cell surface of the parent strain being significantly more hydrophobic. Analysis of the cell wall structure of monolaurin-resistant strains by transmission electron microscopy revealed an increase in the apparent cell wall thickness and contraction of its cytoplasm. Therefore, it can be assumed, that resistance to MAG C12:0 in enterococci is mediated by changes in their cell surface hydrophobicity limiting the access of monolaurin to a potential target in the cytoplasmic membrane and/or in the cytoplasm of the bacterium.

Regardless of reported monolaurin-resistance, the strain *E. faecalis* CCM 4224 was highly sensitive to MAG C12:0 (Figure 8). Within the seven monoacylglycerols tested, monolaurin (MAG C12:0) had the strongest inhibitory effect with total growth inhibition observed at the presence of 100 µg/ml of monolaurin. Even at the lowest concentrations applied

(25 – 50 µg/ml) a significant antibacterial activity was detected as the growth index decreased by more than 80%. Such values are supported by other authors, who recorded MIC values of monolaurin for enterococci at the range 100 – 200 µg/ml [49, 65].
Of other MAGs tested, MAG C10:0 and both MAGs of fatty acids with odd number of carbon atoms (MAG C11:0, MAG C11:1) were successful in inhibiting *E. faecalis* with active concentrations of 100 – 250 µg/ml.

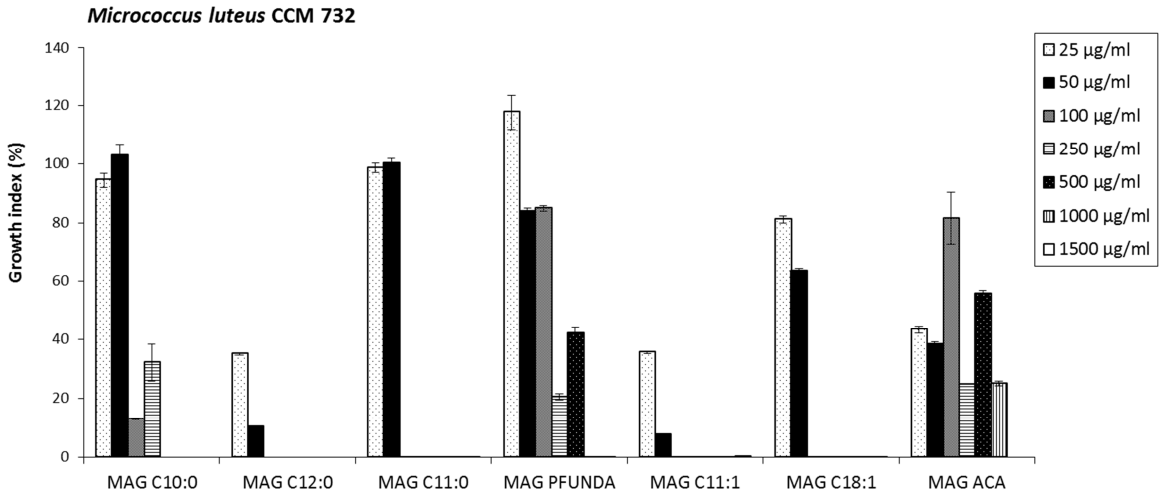
Figure 8. *Growth of Enterococcus faecalis CCM 4224 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



4.3.4. *Micrococcus luteus* CCM 732

Micrococcus luteus was the most sensitive gram-positive species being efficiently inhibited by all monoacylglycerols, except for those that have non-traditional acid bound (Figure 9).
No growth was detected after the application of MAG C12:0, MAG C11:0, MAG C11:1 and monoolein (MAG C18:1) at relatively low concentration 100 µg/ml.
Similar results of antibacterial activity of MAGs with 8 - 16 carbon atoms in fatty acid chain were published by Buňková *et al.* [49]. All MAGs tested were able to stop the growth of *M. luteus* at concentration not exceeding 100 µg/ml and MAG C11:0, MAG C12:0 and MAG C14:0 were active even at 25 µg/ml concentration.

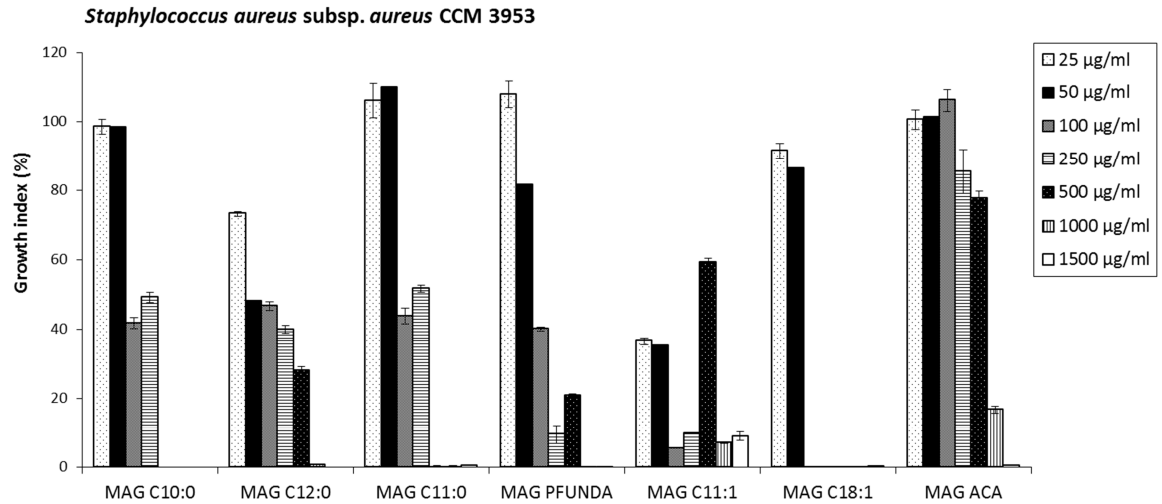
Figure 9. *Growth of Micrococcus luteus CCM 732 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



4.3.5. *Staphylococcus aureus* subsp. *aureus* CCM 3953

In *Staphylococcus aureus* complete growth inhibition occurred usually at higher concentration when compared to other gram-positive bacteria (Figure 10). Surprisingly, the highest inhibitory activity was determined after application of monoolein (MAG C18:1) which had only slight activity in *Bacillus* sp. and *Enterococcus faecalis.*

Figure 10. *Growth of Staphylococcus aureus subsp. aureus CCM 3953 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



Apart from monoolein, satisfactory antibacterial effect had monoacylglycerols with odd number of carbons (MAG C11:0 and MAG C11:1). MIC, i.e. the lowest concentration at which no growth occured, of unsaturated MAG C11:1 against *S. aureus* was higher (1500 µg/ml) than MIC of saturated MAG C11:0

(500 µg/ml). Earlier study by Kabara *et al.* [168] demonstrated MIC for MAG C11:1 (500 mg/ml) that was three times lower in comparison with present study. Lower MIC values for staphylococci were recorded also by Doležalová *et al.* [79] with minimum inhibitory concentrations of MAG C11:0 of 70 µg/ml and for unsaturated form MAG C11:1 the MIC was 140 µg/ml. Authors concluded, that MAG C11:0 and MAG C11:1 are more effective or the same than MAG C10:0 but less effective than MAG C12:0 against gram-positive bacteria. In accordance with this conclusion, our results show comparable activity of MAGs with eleven carbon atoms.

There is a contrast between high activity of monolaurin (MAG C12:0) against staphylococci reported in literature and data obtained for monolaurin in a presented study. Growth index of *S. aureus* was below 50% for monolaurin concentrations 50 - 500 µg/ml, but complete inhibition was not observed until 1500 µg/ml. Conversely, Růžička *et al.* [65] determined MIC value for *S. aureus* to be 35 µg/ml. Similar low MICs are reported by other authors [43, 45]. Significant differences in *S. aureus* sensitivity to MAG C12:0 may be explained by a production of extracellular enzymes. Schlievert *et al.* [70] observed growth inhibition at 20 µg/ml of monolaurin for 8 hours of cultivation. In contrast, by 24 h 20 µg/ml was not inhibitory, by 48 h neither 20 nor 100 µg/ml was inhibitory, and by 96 h no inhibitory activity was seen with MAG C12:0 concentrations of up to 300 µg/ml. The removal of antibacterial activity of monolaurin for *S. aureus* appeared to depend on the production of lipase, which was able to cleave this monoacylglycerol.

Concerning the effect of monolaurin on *S. aureus* cells, there is another interesting point. The activity of monolaurin on staphylococci seems to be more complex, as there are several reports on inhibition of toxin production caused by the presence of MAG C12:0. Moreover, production of hemolysin, toxic shock syndrome toxin 1, and exfoliative toxin A by staphylococci was inhibited at MAG C12:0 concentrations below those necessary to inhibit growth [70].

Within monoacylglycerols selected for this study, MAG of adamantane−1−carboxylic acid was the least efficient and a slight increase in growth index was recorded for MAG ACA concentrations 25 - 100 µg/ml.

4.3.6. Effect of MAGs on growth parameters of grampositive bacteria

In order to assess the effect of monoacylglycerols on bacterial cells more thoroughly, parameters designating the growth of selected bacteria were observed. The data obtained by measuring optical density of bacteria growing in the presence of monoacylglycerols were modelled through a Gompertz equation and the lag-time, the maximum specific growth rate and the maximal value reached were calculated.

Principal changes in growth characteristics of gram-positive bacteria cultivated in nutrient broth supplemented with MAGs can be seen in the following tables that summarize results achieved for *Bacillus cereus.*

As can be seen in Table 3, with increasing concentration of MAGs lag-time extension occurs. For example, in the presence of MAG C10:0 at concentration of 50 µg/ml the length of lag-phase was 0.019 h. At ten times higher concentration 500 µg/ml the lag-time value increased by more than 9 h.

Similarly, the control culture of *B. cereus* had the lag-time value 0.7 h, for the lowest MAG PFUNDA concentration lag-time reached 2.5 h and the increase in lag-time continued until the MAG PFUNDA concentration of 1000 µg/ml, where complete inhibition was achieved.

The prolonged lag-time can be attributed to higher time requirements of bacteria to adaptation to new environmental conditions. On the other hand, low levels of MAG in cultivation medium can lead to a decrease in lag-time values, as can be seen for MAG C10:0. With the lowest concentrations tested, the lag-time value was lower than that of control culture. These findings correspond with the previously mentioned rather beneficial effect of sub-inhibitory MAG concentrations.

Table 3. *The lag time λ (h) of Bacillus cereus CCM 2010 cultivated in the presence of monoacylglycerols estimated by means of Gompertz model*

	Lag time λ (h)							
MAG (µg/ml)	**0**	**25**	**50**	**100**	**250**	**500**	**1000**	**1500**
MAG C10:0	0.221	0.001	0.019	0.024	5.131	9.192	x	x
MAG C12:0	0.726	0.580	0.424	x	x	x	x	x
MAG C11:0	0.001	0.001	0.025	x	x	x	x	x
MAG PFUNDA	0.727	2.554	1.684	1.873	5.605	6.888	x	x
MAG C11:1	0.001	0.001	0.221	0.691	x	x	x	x
MAG C18:1	1.412	1.020	0.793	7.385	9.741	x	x	x
MAG ACA	0.985	0.001	0.400	0.876	1.155	1.252	x	x

x…….no growth detected

Another growth parameter was studied under the influence of monoacylglycerols, the maximum specific growth rate. The values of μ_m calculated for *B. cereus* can be found in Table 4. The maximum specific growth rate of control culture of *B. cereus* was 1.06 h^{-1} and in the presence of MAG C10:0 this value declined to 0.31 h^{-1}. It can be concluded that maximum specific growth rate decreased gradually with an increasing concentration MAGs as well maximal value reached (*A)*. Data on the maximal reached values (*A*) are summarized in Table 5.

Table 4. *The maximum specific growth rate μ_m (h^{-1}) of Bacillus cereus CCM 2010 cultivated in the presence of monoacylglycerols estimated by means of Gompertz model*

	Maximum specific growth rate μ_m (h^{-1})							
MAG (µg/ml)	**0**	**25**	**50**	**100**	**250**	**500**	**1000**	**1500**
MAG C10:0	1.060	1.168	1.051	0.239	0.307	0.310	x	x
MAG C12:0	1.103	1.386	0.619	x	x	x	x	x
MAG C11:0	1.642	1.156	1.197	x	x	x	x	x
MAG PFUNDA	1.406	1.595	1.388	0.984	0.707	0.360	x	x
MAG C11:1	0.896	1.287	1.028	1.439	x	x	x	x
MAG C18:1	0.527	0.492	0.503	0.536	0.523	x	x	x
MAG ACA	1.249	0.712	0.580	0.742	0.574	0.469	x	x

x…….no growth detected

Table 5. *A-value (maximal value reached) of Bacillus cereus CCM 2010 cultivated in the presence of monoacylglycerols estimated by means of Gompertz model*

	Maximal value *A*							
MAG (µg/ml)	**0**	**25**	**50**	**100**	**250**	**500**	**1000**	**1500**
MAG C10:0	5.856	5.792	5.426	4.044	3.442	1.423	x	x
MAG C12:0	5.864	5.165	3.123	x	x	x	x	x
MAG C11:0	5.795	5.796	5.246	x	x	x	x	x
MAG PFUNDA	5.799	5.190	5.570	5.519	5.517	5.527	x	x
MAG C11:1	5.660	5.486	4.091	3.718	x	x	x	x
MAG C18:1	5.432	5.340	4.709	4.300	4.037	x	x	x
MAG ACA	5.946	4.549	3.171	3.280	3.220	2.450	x	x

x…….no growth detected

4.4. EFFECT OF MONOACYLGLYCEROLS ON THE GROWTH OF GRAM-NEGATIVE BACTERIA

According to various literature sources, monoacylglycerols are less effective in inhibiting the growth of gram-negative bacterial species when compared with gram-positive bacteria [49, 63, 65, 78].

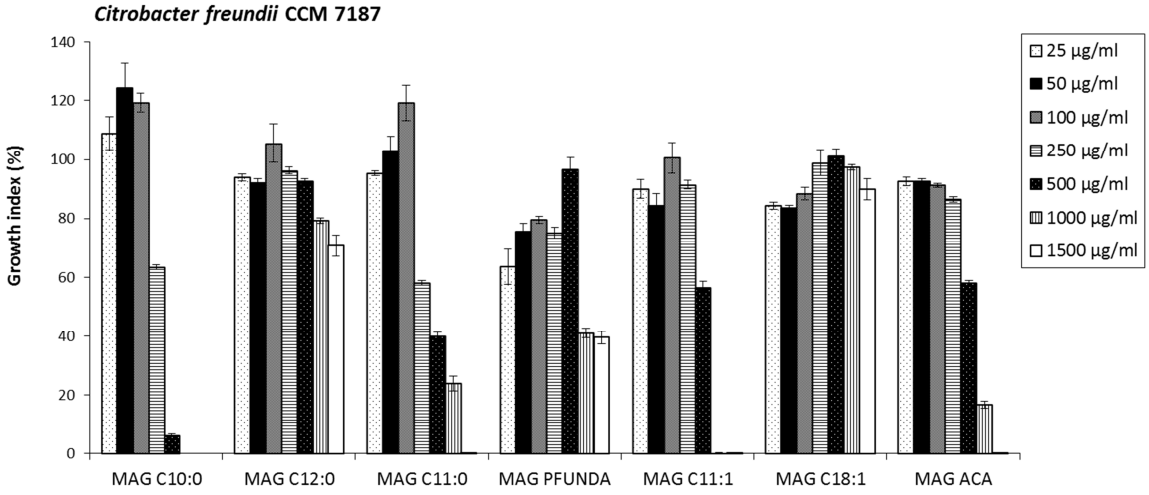
It can be assumed that the major cause lies in a different composition of cell-wall in gram-positive and gram-negative bacteria. In gram-positive bacteria, cytoplasmic membrane is attacked by monoacylglycerols and loses its semi-permeability, which leads to a damage of the bacterial cells. In gram-negative bacteria, it might be the outer membrane that is the place under attack, while there is no damage to the semi-permeability of the cell membrane and thus the bacteria are able to tolerate MAG.

Data on antibacterial activity of seven different monoacylglycerols against gram-negative species are graphically presented in the following section.

4.4.1. Citrobacter freundii CCM 7187

Figure 11 demonstrates lower activity of all MAGs tested on gram-negative species *Citrobacter freundii*.
In most MAGs complete inhibition of growth was observed solely at the highest concentrations 1000 and 1500 µg/ml. Significant supression of growth and multiplying at lower concentration was detected only with monocaprin MAG C10:0 with growth indexes at concentrations 250 and 500 µg/ml 63.34 ± 0.80 and 6.24 ± 0.55, respectively. At concentrations below 250 µg/ml, the growth index rose by approximately 20%.
Holland *et al.* [169] reached similar results. At sub-inhibitory levels of MAG bacterial yields were increased by 15% due to interactions of MAG with plasma membrane with subsequent enhancement of permeability to nutrients.

Figure 11. *Growth of Citrobacter freundii CCM 7187 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*

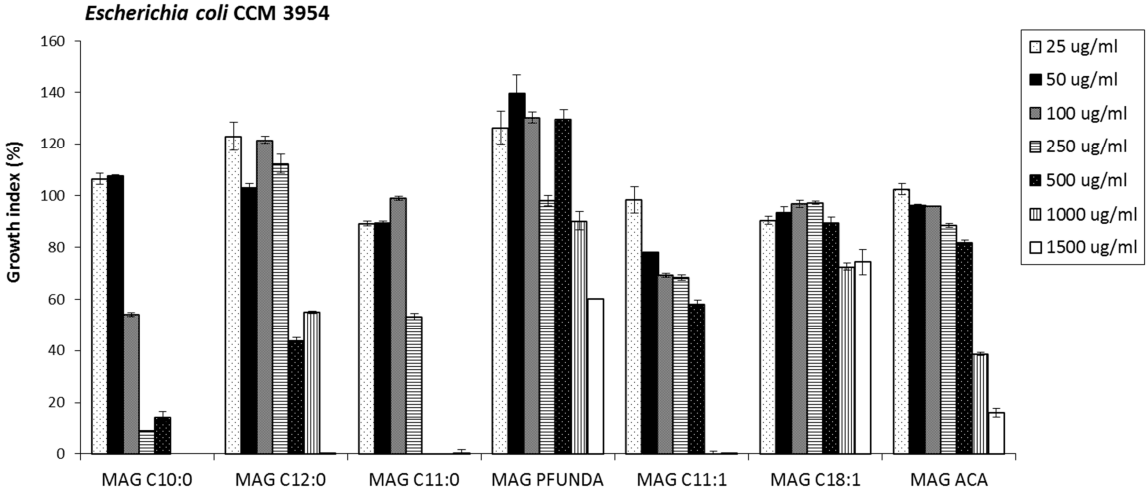


4.4.2. Escherichia coli CCM 3954

Escherichia coli was sensitive to the presence of monoacylglycerols of fatty acids with 10 and 11 carbon atoms with MAG of undecanoic acid being able to cause a complete inhibition at concentration of 500 µg/ml (Figure 12).
MAG of lauric acid (MAG C12:0) did not show a satisfactory inhibition of *E. coli*, although there are literature reports mentioning great activity of this MAG. Altieri *et al.* [63] described the effectiveness of lauric, myristic and palmitic acids and their monoacylglycerols against gram-negative food-borne pathogens including *Escherichia coli* O157:H7. Authors pointed out that the length of the chain was an important element for the antimicrobial effectiveness and lauric acid and monolaurin seemed the most effective compounds in inhibiting *E. coli.* Monolaurin supressed the growth of *E. coli* at considerably low concentration 50 µg/ml, but authors also highlighted that the bioactivity of

MAG C12:0 could be considered quite strong within 10 h and decreased for a prolonged incubation time.

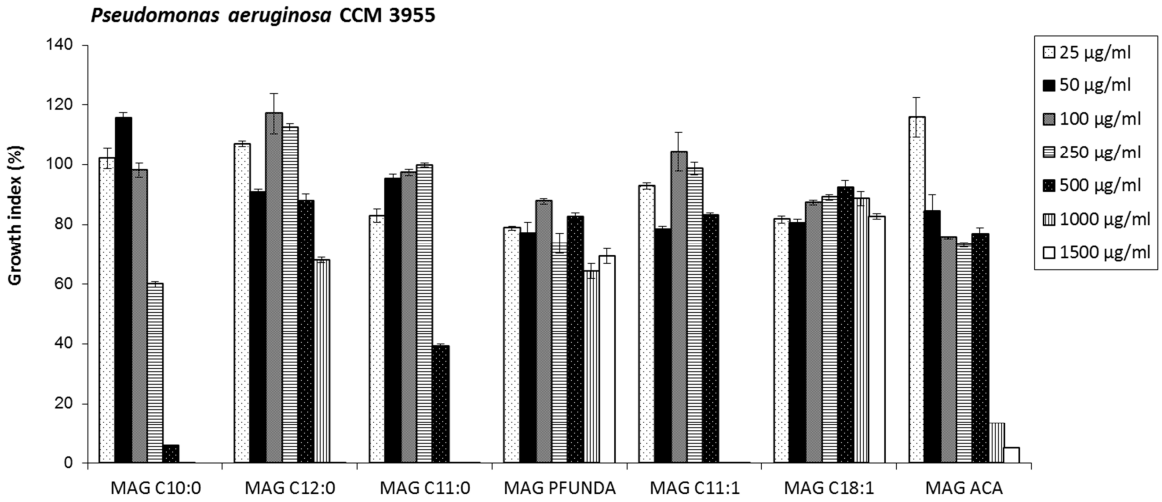
Figure 12. *Growth of Escherichia coli CCM 3954 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



4.4.3. Pseudomonas aeruginosa CCM 3955

The lowest concentration of monoacylglycerols that stopped the growth of *Pseudomonas aeruginosa* was 1000 µg/ml (Figure 13).

Figure 13. *Growth of Pseudomonas aeruginosa CCM 3955 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



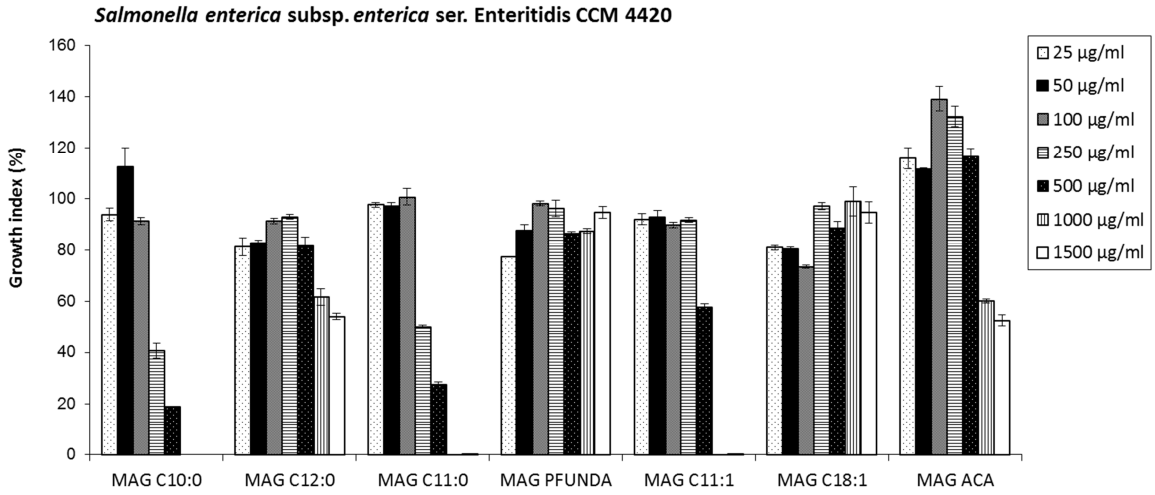
This phenomenon was caused by the same group of MAGs as in *Escherichia coli*, i.e. MAG of capric, undecanoic and undecenoic acid. Application of 500 µg/ml MAG of undecanoic acid (MAG C11:0) to the growth medium led to a decrease of growth index value to 39.01 ± 0.9. Total inhibition also occurred

for MAG of lauric acid when the nutrient broth was supplemented with this MAG to reach a final concentration of 1500 µg/ml.
These results are supported by the study of Růžička *et al.* [65] who did not prove inhibitory action of monocaprin and monolaurin against *P. aeruginosa* even at the highest concentration tested 1000 µg/ml.

4.4.4. *Salmonella enterica* subsp. *enterica* ser. Enteritidis CCM 4420

Results of antibacterial assay with *Salmonella* Enteritidis are closely related to those obtained for *P. aeruginosa.* Monoacylglycerols with 10 and 11 carbons in fatty acid chain caused a complete growth inhibition at a concentration of 1000 µg/ml (Figure 14). Low levels of growth index were recorded for MAG C10:0 at 500 µg/ml (18.85 ± 0.32) and for MAG C11:0 at the same concentration (57.74 ± 1.3). Monoacylglycerol of lauric acid did not have a pronounced effect on *Salmonella* Enteritidis, which is in accordance with other authors who have found no or moderate effect of monolaurin on *Salmonella* sp. [63, 78].

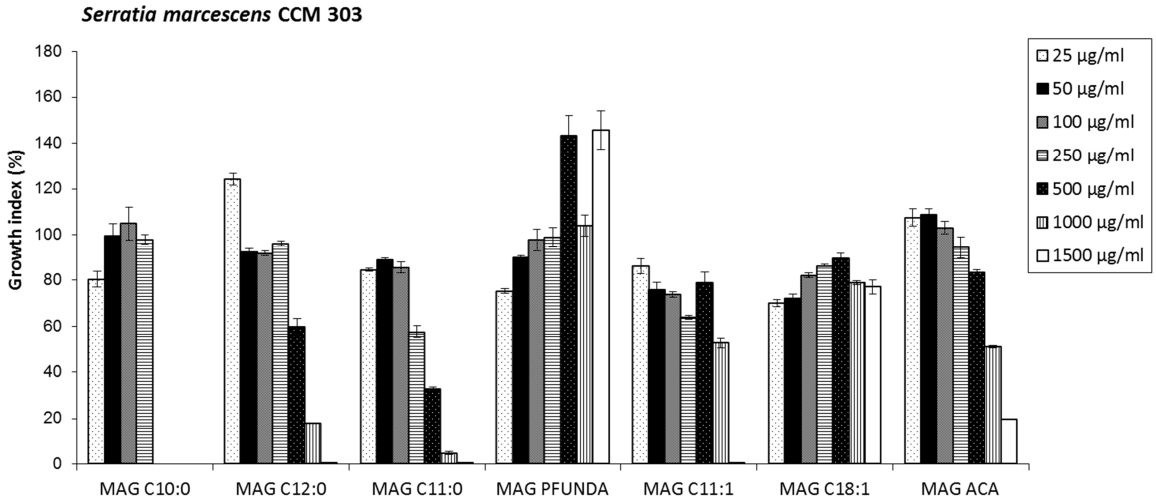
Figure 14. *Growth of Salmonella enterica subsp. enterica ser. Enteritidis CCM 4420 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



4.4.5. *Serratia marcescens* CCM 303

The last gram-negative species *Serratia marcescens* was able to grow relatively unaffected by the presence of monoacylglycerols at concentrations lower than 500 µg/ml. Within monoacylglycerols tested, monoacylglycerols with 10 - 12 carbons in fatty acid chain were the most efficient (Figure 15).

Figure 15. *Growth of Serratia marcescens CCM 303 expressed as growth index after 24 h of cultivation in nutrient broth supplemented with monoacylglycerols*



4.4.6. Effect of MAGs on growth parameters of gram-negative bacteria

Growth characteristics of gram-negative bacteria were obtained by modelling data of optical density using a Gompertz equation. Correspondingly with gram-positive bacteria, determined parameters characterizing the growth of gram-negative species in the presence of MAGs were as follows: the lag-time (λ), the maximum specific growth rate (μ_m) and the maximal value reached (A).

Changes in these growth characteristics caused by monoacylglycerols can be seen in the following tables (Table 6 - 8) that summarize results achieved for *Salmonella enterica* subsp. *enterica* ser. Enteritidis.

The length of the lag-phase of *Salmonella* Enteritidis grew with increasing content of MAGs in cultivation medium as can be seen in Table 6. This increase in lag-time value was more pronounced in MAG C10:0, MAG C11:0 and MAG C18:1 when compared to the other MAGs. At the lowest concentration of MAG C10:0 (25 µg/ml) the period of lag-phase practically did not exist as bacteria were able to start multiplying almost immediately after inoculation. In contrast, when MAG C10:0 was added to medium at 500 µg/ml concentration, bacterial cells required more than 8.6 h to adapt to new environment.

With monoacylglycerol of adamantane-1-carboxylic acid, a steep increase in lag-time was detected as the lag-period prolonged by more than 10 h, but complete growth inhibition by MAG ACA at 1500 µg/ml was not observed.

Table 6. *The lag time λ (h) of Salmonella enterica subsp. enterica ser. Enteritidis CCM 4420 cultivated in the presence of monoacylglycerols estimated by means of Gompertz model*

	Lag-time λ (h)							
MAG (µg/ml)	**0**	**25**	**50**	**100**	**250**	**500**	**1000**	**1500**
MAG C10:0	0.431	0.001	0.001	0.143	0.361	8.636	x	x
MAG C12:0	0.001	0.001	0.807	1.732	2.033	2.496	2.953	3.973
MAG C11:0	0.001	0.069	0.001	1.172	1.828	5.236	x	x
MAG PFUNDA	0.001	0.001	0.001	0.001	0.090	0.874	1.637	2.515
MAG C11:1	0.001	0.001	0.001	0.001	0.001	0.964	x	x
MAG C18:1	0.593	0.511	0.001	7.048	8.570	10.681	9.992	11.325
MAG ACA	0.001	0.001	0.001	0.001	0.001	0.768	5.911	10.819

x…….no growth detected

The values of calculated maximum specific growth rate of *Salmonella* Enteritidis are listed in Table 7. Although the general trend could be observed in gram-positive bacteria with a decreasing growth rate in higher MAG concentration, such generalization cannot be done for gram-negative species. The values of specific growth rate obtained in the presence of different concentrations of MAG were substantially fluctuating. Perhaps the only case of gradual decrease in specific growth rate with increasing MAG concentration could be observed in MAG of adamantane-1-carboxylic acid.

Table 7. *The maximum specific growth rate μ_m (h^{-1}) of Salmonella enterica subsp. enterica ser. Enteritidis CCM 4420 cultivated in the presence of monoacylglycerols estimated by means of Gompertz model*

	Maximum specific growth rate μ_m (h^{-1})							
MAG (µg/ml)	**0**	**25**	**50**	**100**	**250**	**500**	**1000**	**1500**
MAG C10:0	1.656	1.529	1.482	1.208	1.565	0.657	x	x
MAG C12:0	1.159	1.385	1.497	1.597	1.392	1.267	0.822	1.677
MAG C11:0	0.926	0.968	0.997	1.259	1.262	0.311	x	x
MAG PFUNDA	1.356	1.406	1.391	1.389	1.218	1.523	1.947	1.791
MAG C11:1	1.338	1.394	1.349	1.257	1.846	2.070	x	x
MAG C18:1	1.448	0.460	0.301	0.520	0.797	1.003	1.018	1.469
MAG ACA	2.651	2.158	2.291	1.972	1.860	1.723	1.826	1.717

x…….no growth detected

When compared to *A*-values of gram-positive bacteria which were significantly lowered by the addition of MAG to nutrient broth, *A*-values of gram-negative bacteria remained relatively unaffected (Table 8). At the MAG of perfluoroundecanoic acid (MAG PFUNDA) concentrations 25 - 1500 µg/ml *A*–values determined by Gompertz equation ranged from 5.698 to 5.921. Similarly, narrow range (5.438 - 5.774) was observed for MAG of lauric acid and MAG of undecenoic acid (5.253 - 5.695). The only MAG that caused more relevant decrease in *A*-values was MAG C10:0 with *A*-values at 25 µg/ml and 500 µg/ml were 5.789 and 3.966 respectively.

Table 8. *A-value (maximal value reached) of Salmonella enterica subsp. enterica ser. Enteritidis CCM 4420 cultivated in the presence of monoacylglycerols estimated by means of Gompertz model*

	Maximal value *A*							
MAG (µg/ml)	**0**	**25**	**50**	**100**	**250**	**500**	**1000**	**1500**
MAG C10:0	5.789	5.789	5.725	5.538	5.081	3.966	x	x
MAG C12:0	6.004	5.774	5.697	5.600	5.652	5.636	5.546	5.438
MAG C11:0	5.950	5.905	5.831	5.865	5.359	4.907	x	x
MAG PFUNDA	5.961	5.698	5.700	5.759	5.742	5.837	5.874	5.921
MAG C11:1	5.846	5.695	5.524	5.471	5.570	5.253	x	x
MAG C18:1	5.687	6.342	6.486	6.237	5.859	5.684	5.744	5.565
MAG ACA	6.074	6.187	6.151	6.243	6.140	6.051	5.593	5.292

x…….no growth detected

4.5. EFFECT OF MONOACYLGLYCEROLS ON ISOLATED ENDOSPORES

Vegetative cells of bacilli seem to be very sensitive to the antimicrobial activity of monoacylglycerols, especially those with 10 - 12 carbon atoms in fatty acid chain. Since these bacteria are able to form endospores with extraordinary tolerance to various adverse environmental conditions, the effect of MAGs on the behaviour of isolated endospores was examined. Monoacylglycerols selected for these experiments were those which had been previously tested on *Bacillus* sp. vegetative cells and also the concentration range remained unchanged.

Monoacylglycerols have been reported to affect the process of spore germination and outgrowth, however these studies are often focused predominantly on monolaurin [86, 87, 66, 91, 170]. In addition to monolaurin, six other MAGs were examined in order to assess the ability to prevent spore outgrowth. Results are briefly summarized at Table 9 and are expressed by means of minimum inhibitory concentrations (MIC), i.e. the lowest concentration at which no outgrowth was observed.

As expected, the lowest values of MIC were recorded for monolaurin (MAG C12:0). The concentration of MAG C12:0 that prevented spore outgrowth throughout incubation period (7 days) was 25 µg/ml. It should be noted that MIC values of monolaurin for both bacilli vegetative cells were four times higher (see chapters 4.3.1. and 4.3.2.). This finding is in accordance with Ababouch *et al.* [87] who observed similar differences in sensitivity to monolaurin between vegetative cells and endospores of *B. cereus.* A possible cause of lower sensitivity of vegetative cells is their lower hydrophobicity as has been proposed by Chaibi *et al.* [170] who observed the influence of essential oils on vegetative cells and endospores of bacilli and clostridia. Authors found that higher concentration of essential oils were required to supress cell multiplication

as compared with the concentration necessary to inhibit spore germination, i.e. the degradation process by which the dormant state is irreversibly terminated.

Table 9. *Minimum inhibitory concentrations of monoacylglycerols for isolated endospores of Bacillus cereus CCM 2010 and Bacillus subtilis subsp. spizizenii CCM 4062*

	MIC (µg/ml)	
	***B. subtilis* endospores**	***B. cereus* endospores**
MAG C10:0	250	250
MAG C12:0	25	25
MAG C11:0	50	25
MAG PFUNDA	1000	1500
MAG C11:1	100	100
MAG C18.1	500	1000
MAG ACA	250	250

MIC µg/ml (minimum inhibitory concentration) defined as the lowest concentration of MAG that inhibits the outgrowth of endospores for 7 days

Except for monolaurin, MAG C11:0 and MAG C11:1 were also able to inhibit spore outgrowth at low concentrations. The saturated form MAG C11:0 had lower minimum inhibitory concentration for both bacilli strains when compared to unsaturated MAG C11:1. Perfluorinated derivative of MAG C11:0 possessed very low ability in comparison with MAG C11:0.
Relatively low MIC values were also recorded for monocaprin (MAG C10:0) and MAG of adamantane-1-carboxylic acid, which were active at identical concentration 250 µ/ml.

4.6. EFFECT OF MONOACYLGLYCEROLS ON THE GROWTH OF FILAMENTOUS FUNGI

Four species of filamentous fungi were selected to study influence of monoacylglycerols on fungal growth. Growth was observed at the following concentrations of each MAG: 250, 750, 1000 and 1500 µg/ml. Samples were incubated for 14 days at 25 ± 1 °C and colony radii were measured after 7 and 14 days of incubation. Data are expressed as growth index (after 7 or 14 days of cultivation) which compares the growth of fungal strain on agar supplemented with particular MAG concentration with the growth of the same species on agar plates without MAG supplementation. The MIC values were determined from Petri dishes displaying no visible growth with the lowest MAG concentration.
Results of antifungal activity of seven monoacylglycerols are summarized in Table 10. Monoacylglycerols of fatty acids with 10 - 12 carbon atoms as well as MAG of adamantane-1-carboxylic acid were able to prevent the growth of all

micromycetes tested except for *Mucor racemosus*, which proved to be resistant to MAG C11:0.

Table 10. *Minimal inhibitory concentration MIC (µg/ml) of monoacylglycerols for filamentous fungi*

	MIC µg/ml			
	A. alternata	***A. niger***	***M. racemosus***	***P. roqueforti***
MAG C10:0	250	1000	1500	1500
MAG C12:0	750	1500	1500	1500
MAG C11:0	250	1500	> 1500 (NI)	1500
MAG PFUNDA	1500	1500	> 1500 (NI)	> 1500 (NI)
MAG C11:1	750	1000	1500	1500
MAG C18:1	1500	> 1500 (NI)	> 1500 (NI)	> 1500 (NI)
MAG ACA	1000	1000	1000	1500

MIC µg/ml (minimum inhibitory concentration) defined as the lowest concentration of MAG that inhibits the visible growth of an organisms for 14 days; NA not active at concentrations tested

High tolerance of this fungus to MAG activity was also reported by Růžička *et al.* [65] who found that *M. racemosus* was not inhibited by MAG C10:0 and MAG C12:0 at 1000 and 750 µg/ml, respectively. Doležalová *et al.* [79] have reported resistance of *M. racemosus* to monoacylglycerols of undecanoic and undecenoic acid which were not active at 1000 µg/ml. In this study, MAG C11:0 did not suppress the growth of this fungus at 1500 µg/ml, but the same concentration of MAG C11:1 inhibited visible growth of an organism for 14 days. Within all MAGs tested, only MAG ACA had the MIC value for *M. racemosus* lower than 1500 µg/ml.

Alternaria alternata was the most sensitive species with relatively low MIC values 250 - 750 µg/ml at the presence of MAGs with 10 - 12 carbon atoms. Sensitivity of this fungus to MAGs is supported by literature [65, 68, 79]. *Aspergillus niger* was inhibited by all tested monoacylglycerols except for MAG of oleic acid (MAG C18:1).

Penicillium roqueforti seems to be relatively tolerant to the presence of monoacylglycerols in environment (Figure 16). MAG of oleic and perfluoroundecanoic acid did not cause a total growth suppression and for other MAGs tested the MIC value was not recorded until the highest concentration of 1500 µg/ml. Buňková and coworkers [68] observed a strong reduction of colony size in some penicillia including *P. roqueforti* at 130 µg/ml concentration of monolaurin and monocaprin. Figures 16 and 17 show the growth index values of *P. roqueforti* determined after 7 and 14 of cultivation on agar plates supplemented with MAGs. Although total growth inhibition was observed merely at the highest concentration tested, growth indexes at 750 µg/ml concentration of all MAGs did not exceed 40%. For MAG C11:0 and MAG C11:1 this inhibitory action remained even at prolonged cultivation time (14 days).

Figure 16. *Growth of Penicillium roqueforti expressed as growth index after 7 days of cultivation on Fungal agar supplemented with monoacylglycerols*

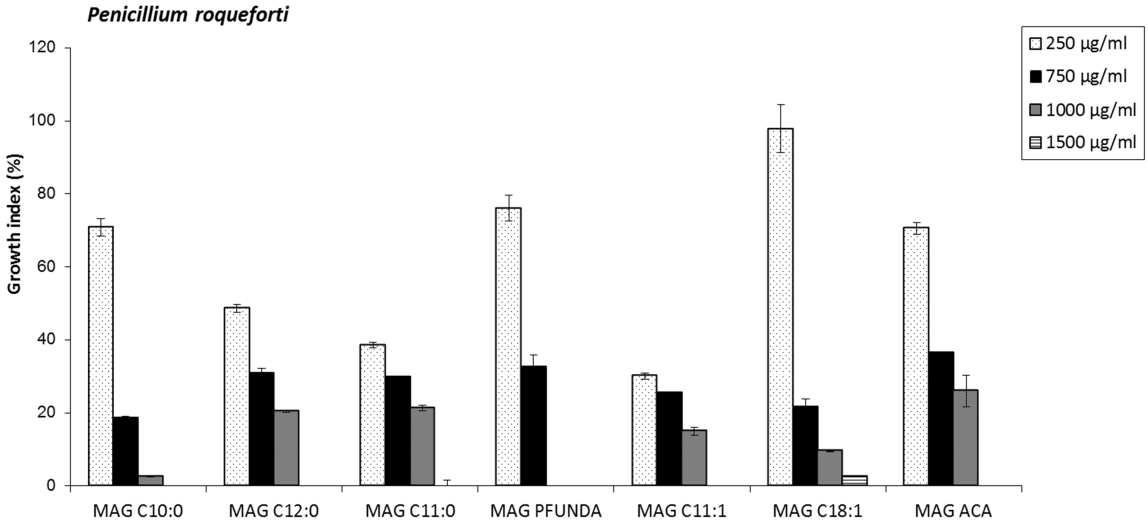
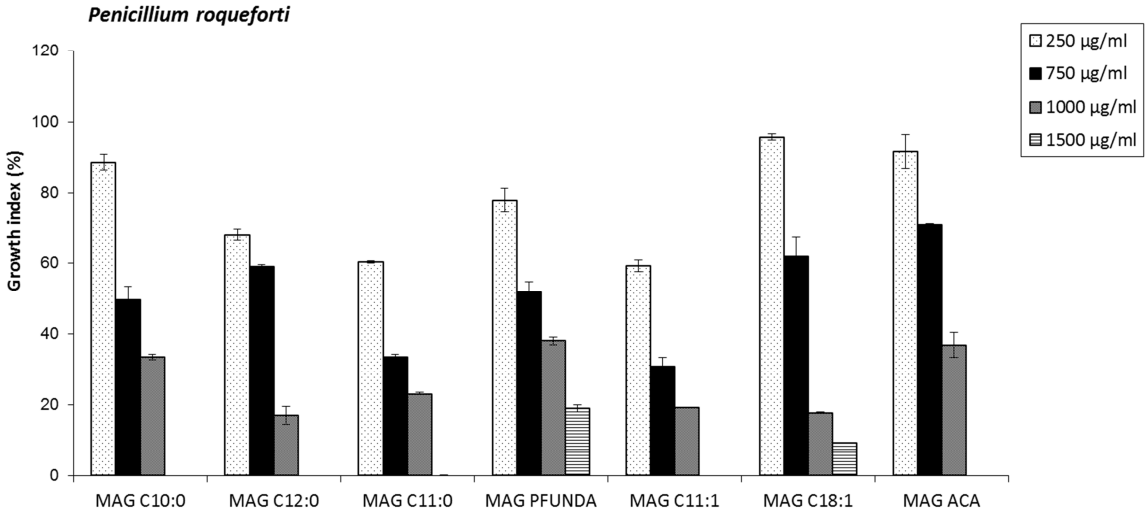


Figure 17. *Growth of Penicillium roqueforti expressed as growth index after 14 days of cultivation on Fungal agar supplemented with monoacylglycerols*



4.7. EFFECT OF MONOACYLGLYCEROLS ON SPORE-FORMING BACTERIA IN PROCESSED CHEESE SAMPLES

In general, microbiological safety of processed cheese is ensured by the melting process that requires temperatures of 90 - 100 °C with the total melting time between 5 - 15 min. This procedure can be considered as pasteurization and if the right production and storage principles are followed, microbial contamination of processed cheese is limited to those organisms that can survive such treatment. From a microbiological point of view, the biggest contamination problem of processed cheese is initiated by spore-forming rod-shaped bacteria of genera *Bacillus* and *Clostridium.* One of the major aims of the presented thesis was to examine an influence of some factors on proliferation of these spore-forming microorganisms in processed cheese. Within these factors, the thesis deals mainly with the activity of monoacylglycerols that can serve as antimicrobial agents as well as they can improve some textural properties of processed cheese, thus are believed to be promising as potential additives.

Based on the results of *in vitro* evaluation of antimicrobial activity of seven monoacylglycerols on wide range of food-borne pathogens or spoilage bacteria, four monoacylglycerols with the highest antimicrobial activity were selected. These MAGs were included in the composition of ingredients and processed cheese samples were produced by procedure consisted of the following steps: blending ingredients with water, processing by heating and shearing of the blend, homogenization of the molten blend and finally hot packaging and cooling. Subsequently, samples were inoculated with each of the selected microbial target and stored at 6 ± 2 °C for 140 days. During storage, cheese samples were removed from incubation at selected time intervals and microbiologically analyzed. Data were expressed as log CFU per gram of the sample and plotted against time to provide a survival curves.

Monoacylglycerols selected for this experiment were:

- monoacylglycerol of undecanoic acid (MAG C11:0)
- monoacylglycerol of undecenoic acid (MAG C11:1)
- monoacylglycerol of lauric acid (MAG C12:0)
- monoacylglycerol of adamantane-1-carboxylic acid (MAG ACA)

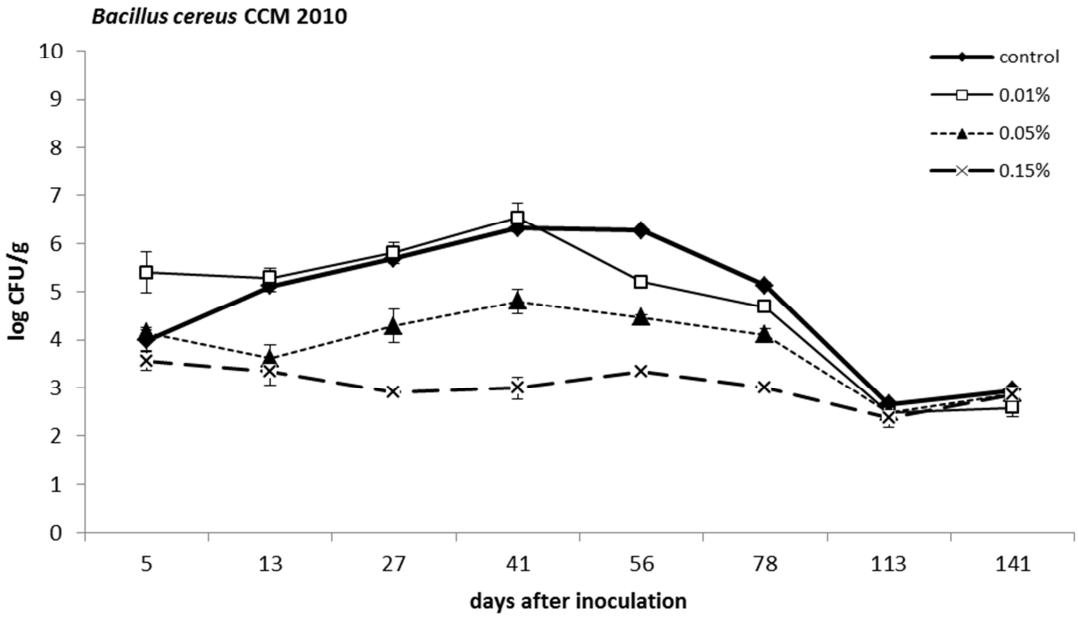
Monoacylglycerols with 11 carbon atoms in fatty acid chain (MAG C11:0 and MAG C11:1) were selected due to their high activity against nearly all microorganisms tested *in vitro*. MAG C12:0 also proved good antimicrobial potential *in vitro* and according to literature sources is considered to be the most efficient monoacylglycerol. MAG ACA, although not very convincing in inhibition of gram-positive bacteria nor filamentous fungi, caused a great prolongation of lag-time in gram-negative bacteria and had very low MIC values for isolated endospores.

4.7.1.Effect of monoacylglycerol of undecanoic acid (MAG C11:0) on spore-forming bacteria in processed cheese samples

Influence of MAG C11:0 was examined in processed cheese samples with 50% fat in dry matter content supplemented with MAG C11:0 at concentrations 0.01, 0.05 and 0.15% (w/w). Growth curves obtained for *Bacillus cereus* and *Bacillus subtilis* are presented in Figure 18 and 19.

Both bacilli were not significantly affected by the addition of MAG C11:0 at the lowest concentration 0.01%. Growth curves for 0.01% concentration almost exactly replicated curves of the control culture growing in processed cheese samples without MAG C11:0.

Fixure 18. *Growth and survival of Bacillus cereus CCM 2010 inoculated into processed cheese supplemented with monoacylglycerol of undecanoic acid (MAG C11:0)*

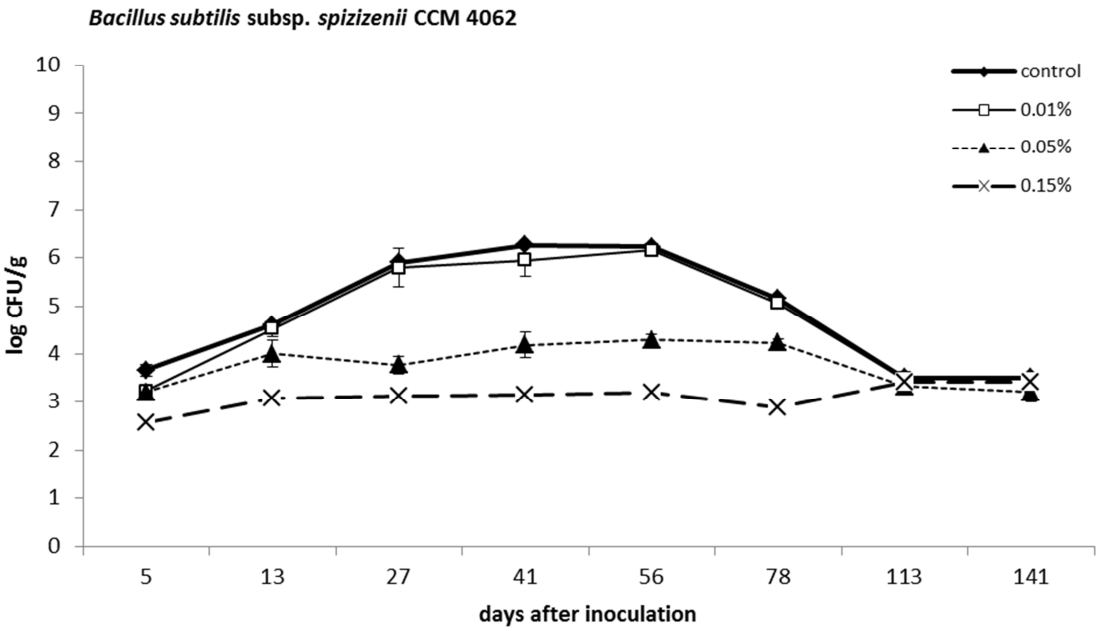


At 0.05% concentration of MAG C11:0 a partial growth inhibition was observed in both strains of *Bacillus* sp. Number of cells in these samples decreased by approximately 2 log CFU/g related to control and this effect remained noticeable for 80 days of storage.

Processed cheese samples enriched with MAG C11:0 at concentration of 0.15% maintained number of cells of *B. cereus* and *B. subtilis* at the same level by the end of the storage period. Thus, in the presence of MAG C11:0 at a concentration of 0.15% the growth and multiplying of bacilli is prevented and lower concentration 0.05% lead to a partial inhibition.

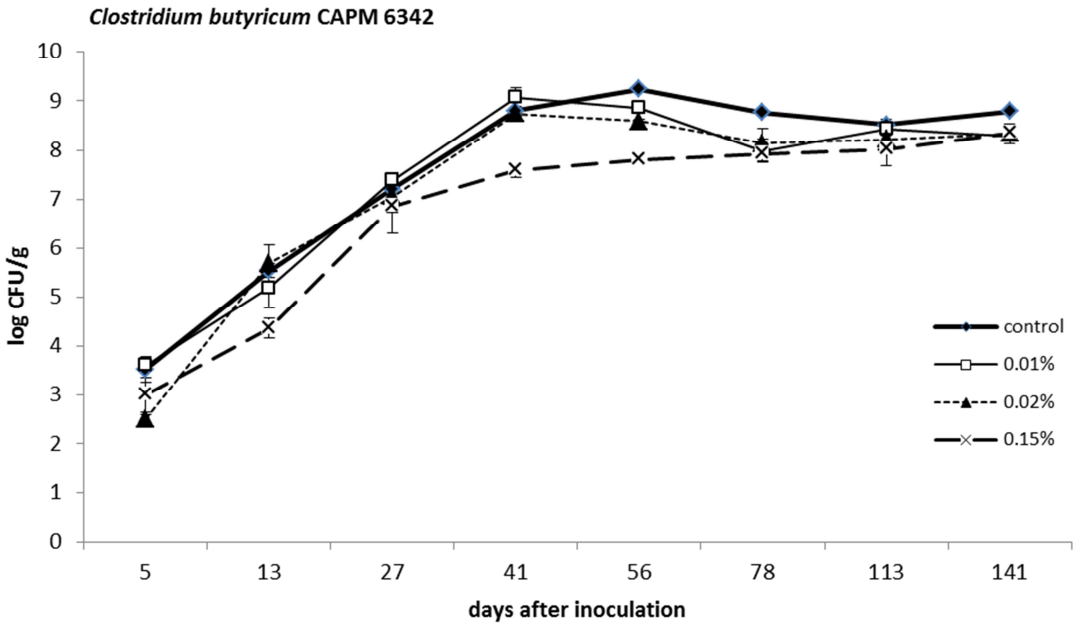
These results suggest that a sensitivity of *Bacillus* sp. to MAG of undecanoic acid is not limited to cultivation media, but can be achieved also in real conditions of foods.

Figure 19. *Growth and survival of Bacillus subtilis subsp. spizizenii CCM 4062 inoculated into processed cheese supplemented with monoacylglycerol of undecanoic acid (MAG C11:0)*



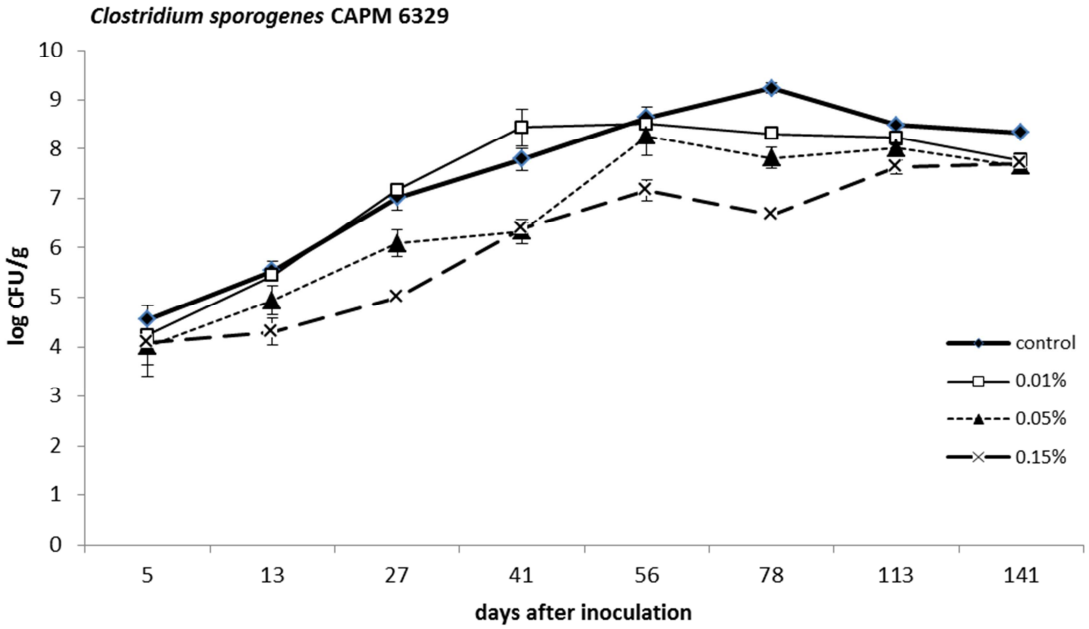
When compared to bacilli, clostridia were shown to be more resistant to MAG C11:0 antimicrobial action as only partial growth inhibition occurred in cheese samples. Number of cells of *Clostridium butyricum* increased gradually during the storage period in all processed cheese samples regardless of monoacylglycerol addition (Figure 20).

Figure 20. *Growth and survival of Clostridium butyricum CAPM 6342 inoculated into processed cheese supplemented with monoacylglycerol of undecanoic acid (MAG C11:0)*



Growth of *Clostridium sporogenes* in processed cheese samples supplemented with MAG C11:0 is presented in Figure 21. *Cl. sporogenes* appeared to be slightly more sensitive to this monoacylglycerol than *Cl. butyricum.* The CFU value declined at 0.15% concentration of MAG by 1.5 log units. Nevertheless, considerable growth reduction was not observed even at the highest concentration of MAG C11:0.

Figure 21. *Growth and survival of Clostridium sporogenes CAPM 6342 inoculated into processed cheese supplemented with monoacylglycerol of undecanoic acid (MAG C11:0)*



4.7.2. Effect of monoacylglycerol of undecenoic acid (MAG C11:1) on spore-forming bacteria in processed cheese samples

On the basis of *in vitro* data, it could be predicted that MAG of undecenoic acid would have lower ability to inhibit the growth of spore-forming bacteria. Minimum inhibitory concentration recorded for MAG C11:1 was higher than those of MAG C11:0 at least for *Bacillus* sp. vegetative cells and spores.

According to Doležalová *et al.* [79] the unsaturated analogue MAG C11:1 exhibited more prominent antimicrobial properties against bacteria and fungi when compared to MAG C11:0. Their findings are in contrast to *in vitro* obtained results of MAG C11:0 and MAG C11:1 activity reported in this thesis. However the differences in activity of these MAGs were not substantial as both tested MAGs showed satisfactory inhibition activity against bacteria causing alimentary diseases even at low concentrations.

Despite the initial assumption, the activity of unsaturated analogue resembled the activity of saturated form of MAG with 11 carbon atoms. With MAG C11:1 at concentrations of 0.01% and 0.05% partial growth inhibition of both bacilli occurred with a decrease in lof CFU/g values. Monoacylglycerol of undecenoic

acid at 0.15% concentration prevented bacilli from growing and CFU values remained at the initial value.
Growth curves of *Bacillus cereus* and *Bacillus subtilis* are shown below in Figure 22 and Figure 23, respectively.

Figure 22. *Growth and survival of Bacillus cereus CCM 2010 inoculated into processed cheese supplemented with monoacylglycerol of undecenoic acid (MAG C11:1)*

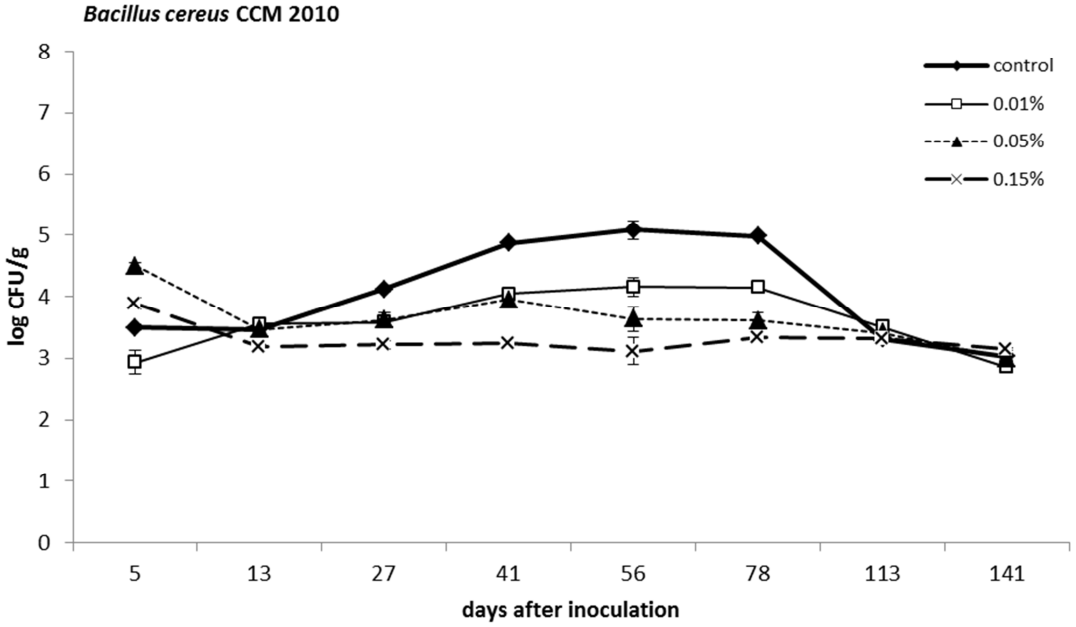
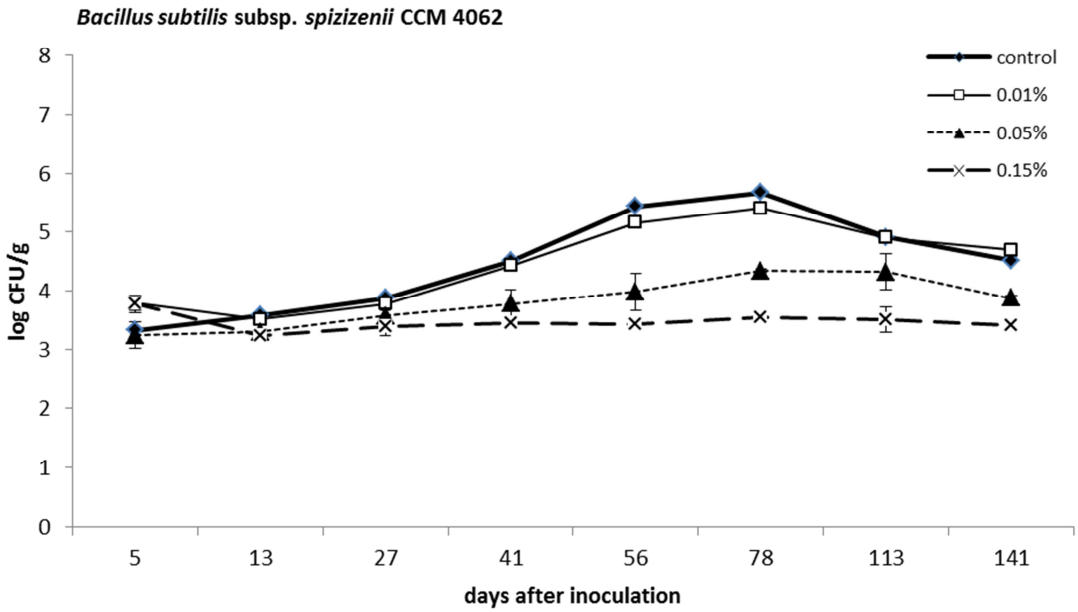
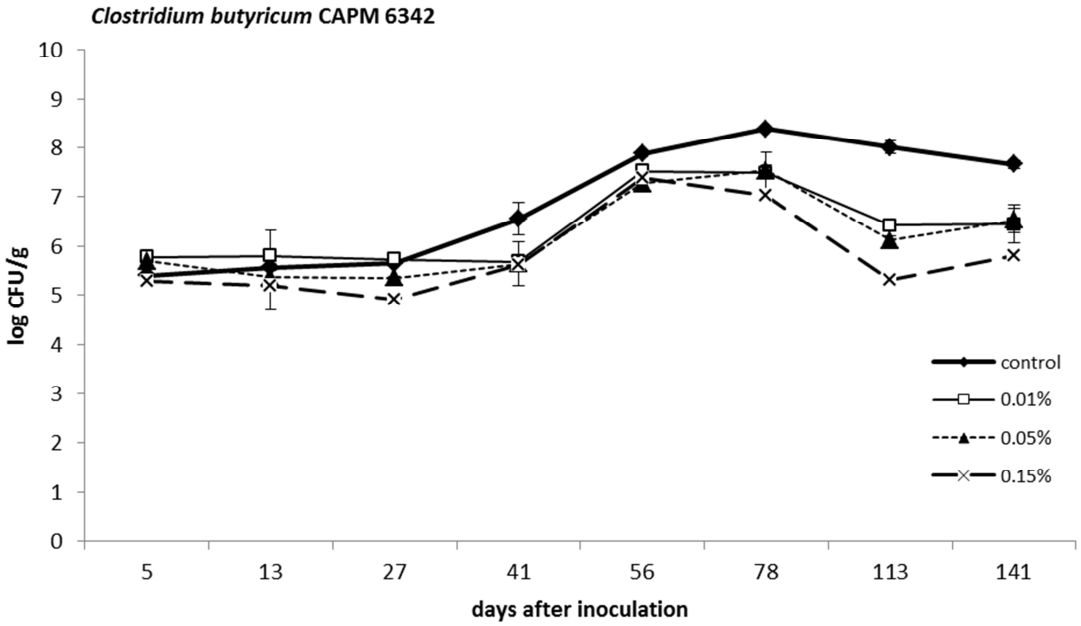


Figure 23. *Growth and survival of Bacillus subtilis subsp. spizizenii CCM 4062 inoculated into processed cheese supplemented with monoacylglycerol of undecenoic acid (MAG C11:1)*



As can be seen in Figure 24, *Clostridium butyricum* cell numbers were reduced under the influence of MAG C11:1, although a complete suppression of growth was not achieved. Decrease in CFU values observed at MAG C11:1 concentration 0.01 and 0.05% were almost identical with approximately 2 log drop at the end of storage period compared to control. Moro pronounced differences in log CFU/g values were detected for the highest concentration of MAG. After 113 days of storage log CFU/g for processed cheese samples with 0.15% concentration was 5.31 ± 0.08 and control processed cheese samples reached 8.01 ± 0.13 log CFU/g. Despite this obvious inhibitory effect, MAG C11:1 was not able to stop clostridia from growing in the same manner as in *Bacillus* sp.

Figure 24. *Growth and survival of Clostridium butyricum CAPM 6342 inoculated into processed cheese supplemented with monoacylglycerol of undecenoic acid (MAG C11:1)*

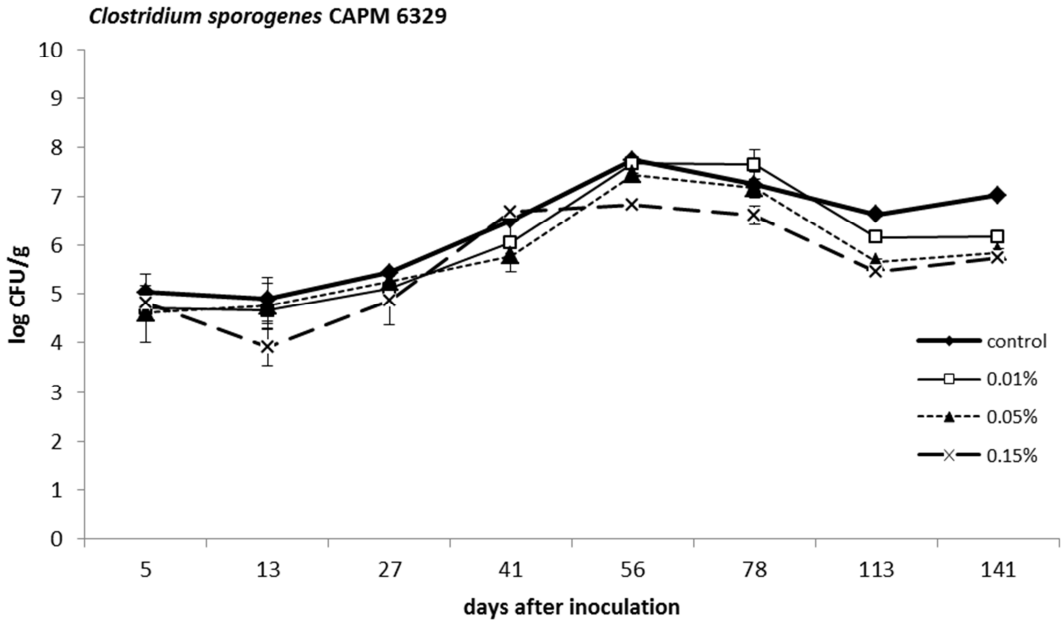


Growth of another *Clostridium* species *Clostridium sporogenes* appeared to be less affected by MAG of undecenoic acid with cell number reduction not exceeding 1.3 log decline (Figure 25).

Based on the above mentioned results of inhibitory activity of MAG C11:0 and MAG C11:1, higher ability to tolerate monoacylglycerols was noticed in clostridia compared to bacilli. As reported by Chaibi *et al.* [170] vegetative cells of *Bacillus cereus* were more sensitive to essential oils than vegetative cells of *Clostridium botulinum.* Higher sensitivity was also noticed for endospores of bacilli whereas the outgrowth process in spores of *Cl. botulinum* was resistant to essential oils which might be probably caused by anaerobic nature of *Cl. botulinum.* This hypothesis is supported by Ababouch and co-workers [87] who demonstrated that inhibition of *B. cereus* outgrowth by monolaurin was

caused by an inhibition of oxygen consumption, while the outgrowth of anaerobic clostridia remained unaffected [91].

Figure 25. *Growth and survival of Clostridium sporogenes CAPM 6342 inoculated into processed cheese supplemented with monoacylglycerol of undecenoic acid (MAG C11:1)*



4.7.3. Effect of monoacylglycerol of lauric acid (MAG C12:0) on spore-forming bacteria in processed cheese samples

Monolaurin (MAG C12:0) is reported to possess wide range of antimicrobial efficacy, especially against gram-positive bacteria including those that are able to form endospores [43, 49, 65, 91, 170].

In nutrient broth the activity of monolaurin against *B. cereus* and *B. subtilis* was found to be very strong and MIC values of monolaurin for bacilli were low. Unfortunately, inhibitory action of MAG C12:0 in real environment of processed cheese was not satisfactory as illustrated by growth curves in Figures 26 and 27. Lower activity of monolaurin in processed cheese might be explained by interactions with processed cheese components. Generally, the activity of fatty acids and their derivatives against bacteria could be decreased or completely blocked as it is affected by the presence of starch, proteins such as serum albumin, lipids such as phospholipids, and other surface-active agents such as cholesterol [12].

Neither bacilli nor clostridia were prevented from growing in processed cheese supplemented with monolaurin. According to Mansour and Milliere [165] who focused on inhibitory activity of monolaurin and nisin against bacilli in milk, synergistic effect between monolaurin and nisin occurred in milk. After addition

of nisin, the activity of monolaurin was enhanced leading to bactericidal effect and total inhibition of bacilli.

Figure 26. *Growth and survival of Bacillus cereus CCM 2010 inoculated into processed cheese supplemented with monoacylglycerol of lauric acid (MAG C12:0)*

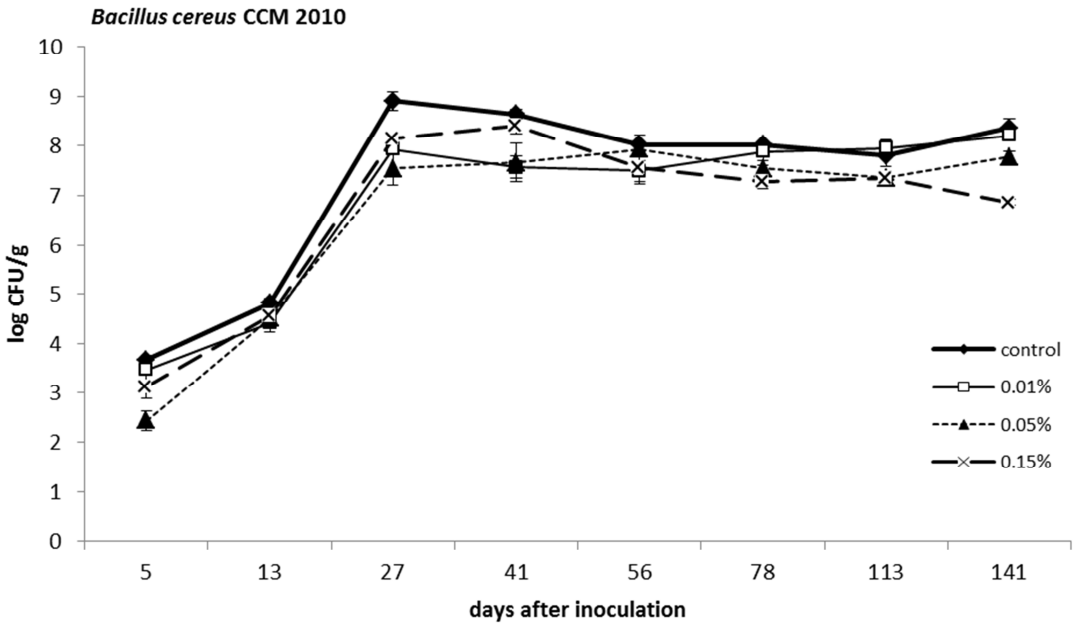
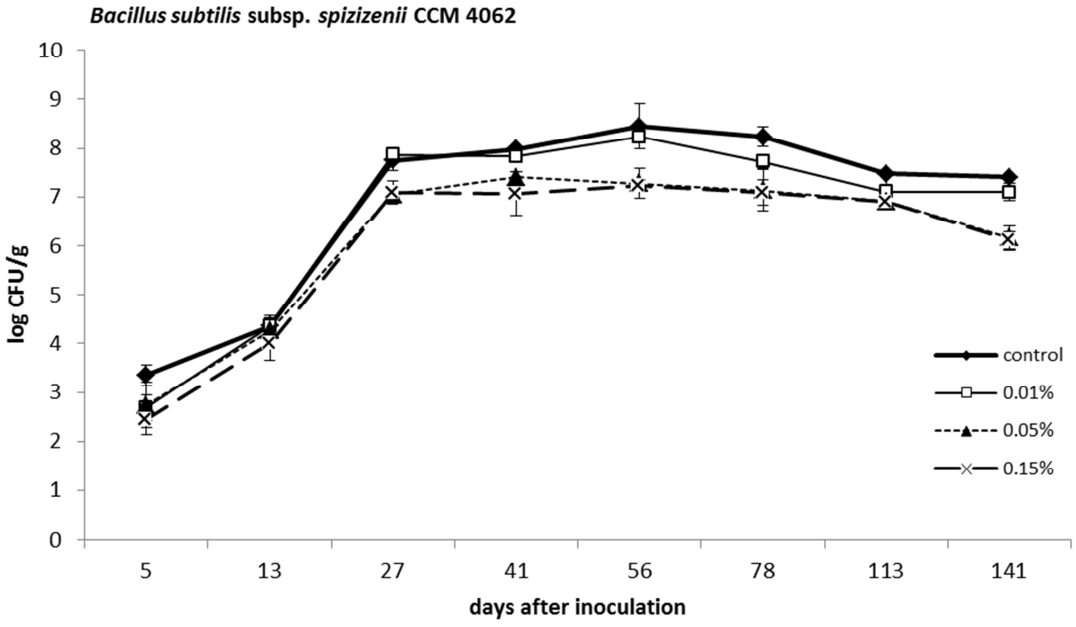


Figure 27. *Growth and survival of Bacillus subtilis subsp. spizizenii CCM 4062 inoculated into processed cheese supplemented with monoacylglycerol of lauric acid (MAG C12:0)*



Figures 28 and 29 show the influence of MAG C12:0 on clostridia inoculated into processed cheese samples. Total inhibition was not observed even at the highest concentration tested, but a partial inhibition was noticed for monolaurin at all three concentrations tested. *Clostridium butyricum* was more sensitive to monolaurin in comparison with *Clostridium sporogenes.*

Figure 28. *Growth and survival of Clostridium butyricum CAPM 6342 inoculated into processed cheese supplemented with monoacylglycerol of lauric acid (MAG C12:0)*

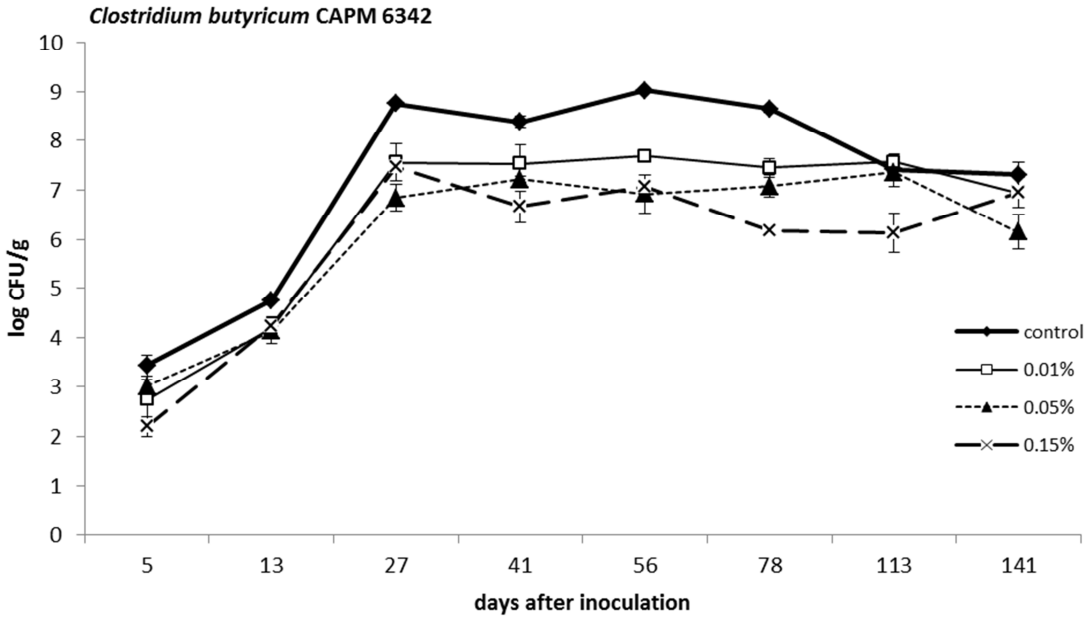
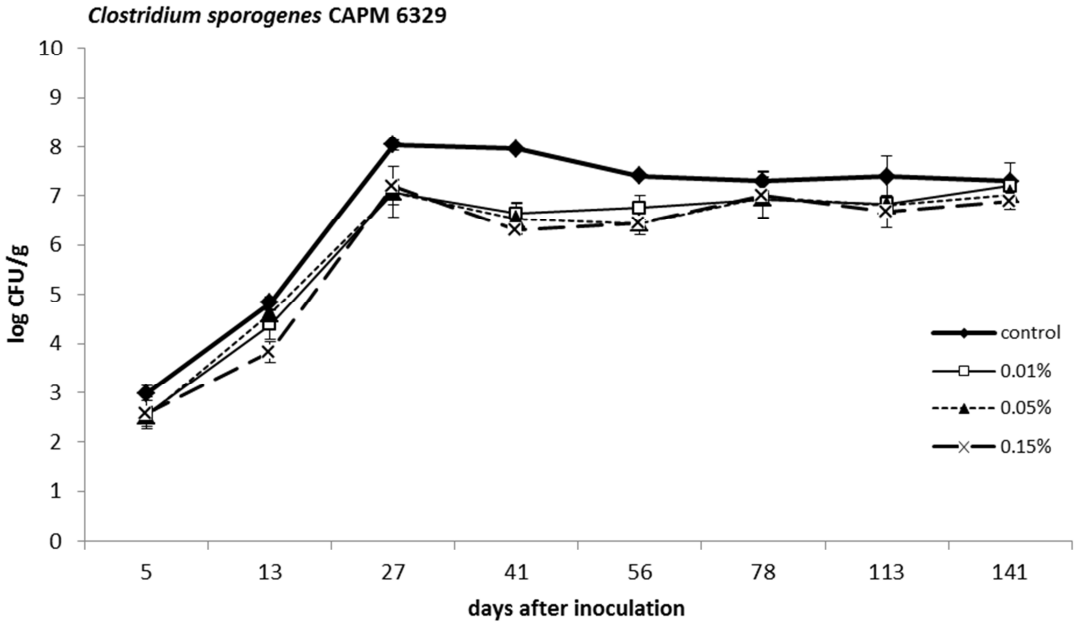


Figure 29. *Growth and survival of Clostridium sporogenes CAPM 6342 inoculated into processed cheese supplemented with monoacylglycerol of lauric acid (MAG C12:0)*



4.7.4. Effect of monoacylglycerol of adamantane-1-carboxylic acid (MAG ACA) on spore-forming bacteria in processed cheese samples

Compounds containing adamantane skeleton first attracted attention in 1964 after the publication of studies dealing with antiviral activity of 1−adamantylamine [171]. This discovery triggered a massive surge of interest in these and similar compounds, which led to the discovery of many interesting biological activities of adamantane derivatives. The most known clinical use is as antiviral drugs amantadine and rimantadine. Adamantane derivatives also exhibit anticancer activity [172, 173].

Antimicrobial actions of compounds containing adamantane moiety have been published as well. Phthalimide derivatives of adamantane showed a very strong antibacterial activity, minimal inhibitory concentrations for these compounds against *Staphylococcus aureus* and *Micrococcus flavus* were comparable with that of clinically used antibiotics (1-0.02 µg/ml) [174]. Analogues of 4-(1-adamantyl)-2-quinolinecarbohydrazide exhibited excellent antimycobacterial activities in the range of 6.25–3.125 µg/mL against drug-sensitive and drug-resistant *Mycobacterium tuberculosis* [175]. Other adamantane derivatives had a broad-spectrum effect on certain gram-positive and gram-negative bacteria, acid-fast bacteria, yeasts and filamentous fungi [176-181].

Since both substances, monoacylglycerols and derivatives of adamantane, have been previously shown to be active against microorganisms, the molecule that might benefit from the properties of both substances has been proposed in order to find a new effective antibacterial agent.

Although the activity of MAG containing adamantane skeleton (MAG ACA) did not show as high *in vitro* activity as did monoacylglycerols with 10 - 12 carbon atoms in fatty acid chain, its inhibitory action against spore-forming bacteria in processed cheese was noteworthy.

MAG ACA at the highest tested concentration 0.15% caused a great reduction in microbial counts for both strains of bacilli as shown in Figure 30 and 31. The obtained log CFU/g values fluctuated near the initial value throughout the storage period.

Clostridium butyricum inoculated to processed cheese samples with MAG ACA content of 0.15% was similarly affected although the log CFU/g values raised by approximately 1 log within incubation (Figure 32).

In contrast, *Clostridium sporogenes* was poorly influenced by the presence of monoacylglycerol of adamantine-1 carboxylic acid as can be seen in Figure 33.

Figure 30. *Growth and survival of Bacillus cereus CCM 2010 inoculated into processed cheese supplemented with monoacylglycerol of adamantane-1-carboxylic acid (MAG ACA)*

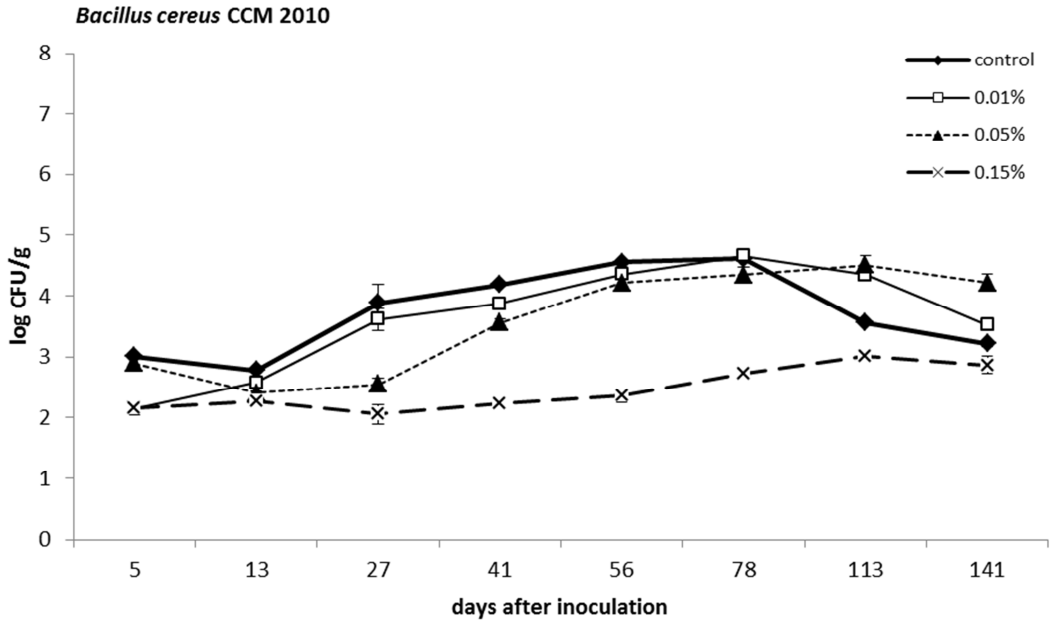


Figure 31. *Growth and survival of Bacillus subtilis subsp. spizizenii CCM 4062 inoculated into processed cheese supplemented with monoacylglycerol of adamantane-1-carboxylic acid (MAG ACA)*

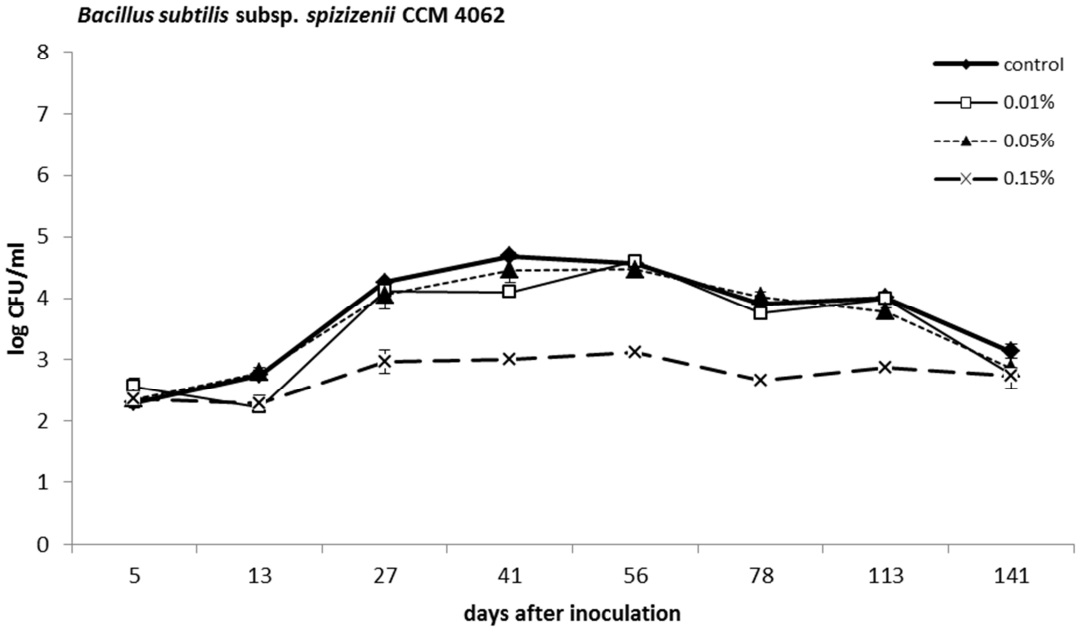


Figure 32. *Growth and survival of Clostridium butyricum CAPM 6342 inoculated into processed cheese supplemented with monoacylglycerol of adamantane-1-carboxylic acid (MAG ACA)*

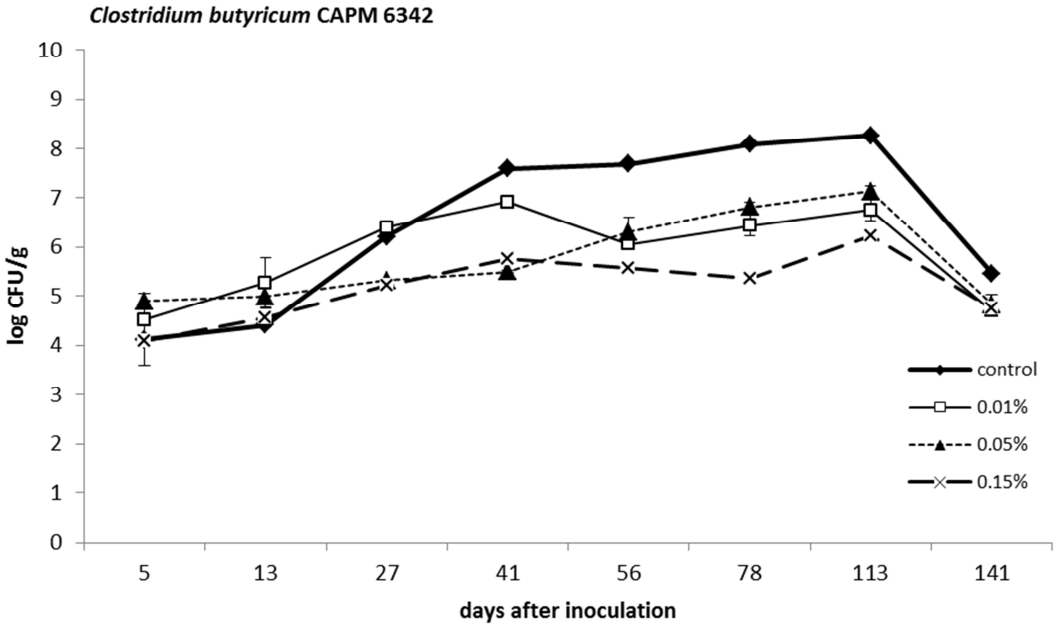
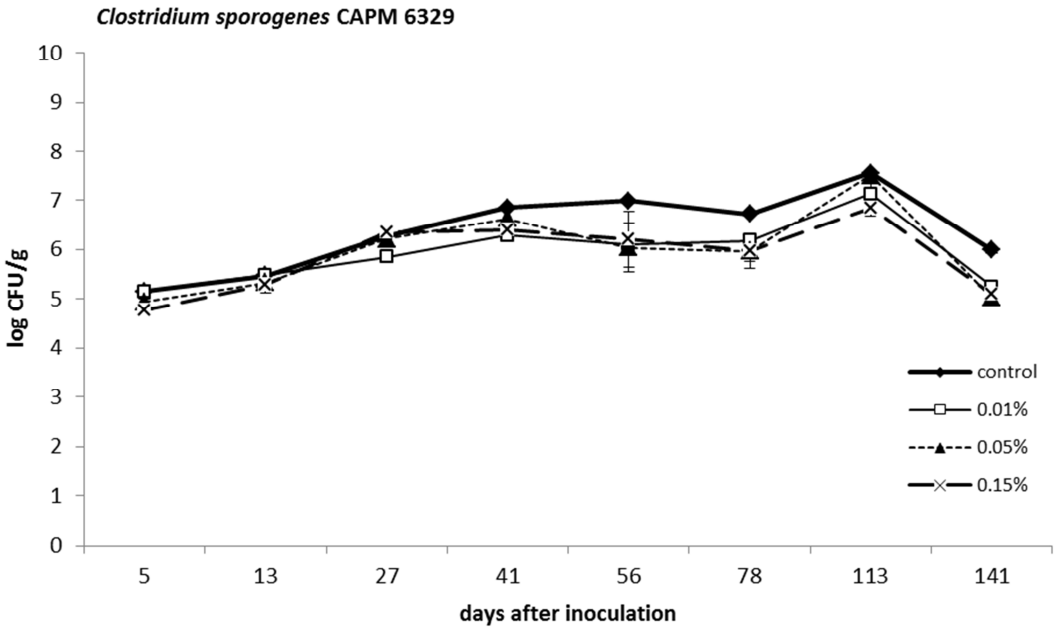


Figure 33. *Growth and survival of Clostridium sporogenes CAPM 6342 inoculated into processed cheese supplemented with monoacylglycerol of adamantane-1-carboxylic acid (MAG ACA)*



4.8. EFFECT OF FAT CONTENT OF PROCESSED CHEESE ON THE GROWTH OF SPORE-FORMING BACTERIA

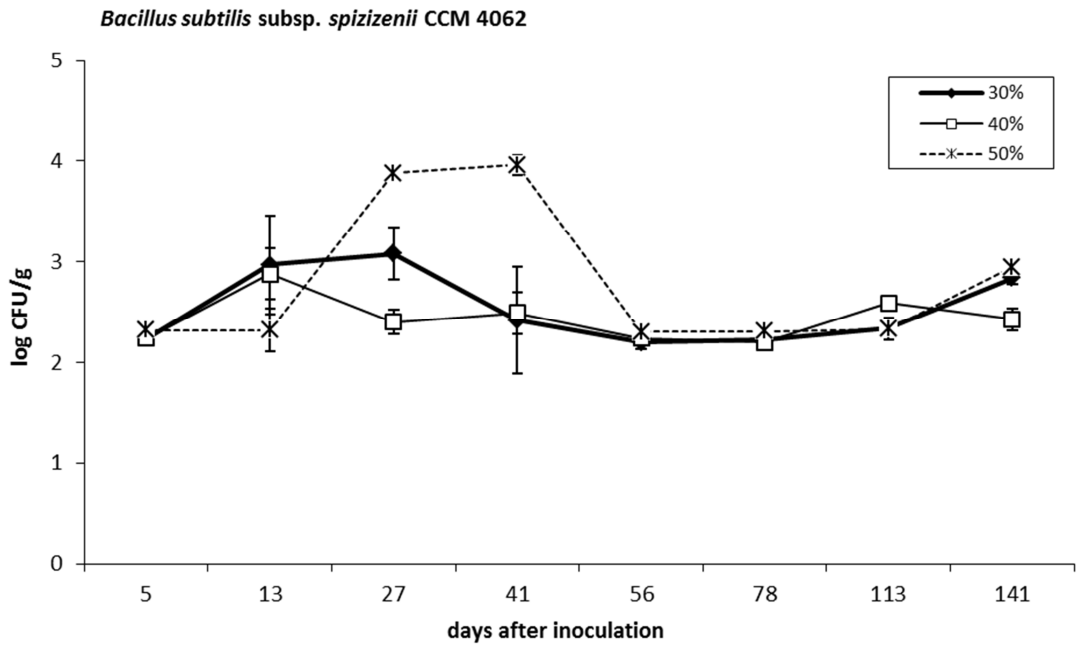
In order to examine the influence of fat level on microbial surivival, processed cheese samples with fat in dry matter content of 30%, 40% and 50% were produced and inoculated with spore-forming bacilli and clostridia. Correspondingly with processed cheese samples supplemented with monoacylglycerols, samples differing in fat content were stored for 140 days and microbiological analysis was carried out at the same time intervals.

According to various literature sources, cheese with lower fat content can be considered a less hospitable environment for survival of various microorganisms [76, 95, 97, 143]. Spore-forming microorganisms are often suppressed in their multiplication, or at least their growth is significantly delayed in reduced-fat cheese [95, 97, 109]. Moreover, the production of microbial toxins in reduced-fat or fat-free processed cheese products may be restricted or prevented entirely [142].

As far as *Bacillus* sp. is concerned, the results are similar to above mentioned information on the survival and growth of spore-forming bacteria in cheese with low fat level.

The microbial growth curves for *Bacillus subtilis* subsp. *spizizenii* in processed cheese samples differing in fat level are shown in Figure 34.

Figure 34. *Effect of fat content on growth and survival of Bacillus subtilis subsp. spizizenii CCM 4062 inoculated into processed cheese*



Growth patterns of *B. subtilis* in processed cheese with 30 and 40% fat in dry matter were similar throughout the storage period. In processed cheese samples with higher fat level 50%, a sharp increase in cell population was noted after two weeks of storage. Higher population levels in cheese with higher fat content lasted another 40 days of storage. In the second half of storage period, no significant differences in cell number were observed among samples with 30, 40 and 50% fat in dry matter.

In contrast with *Bacillus* sp., the fat level did not influence the growth and survival of *Clostridium butyricum* and *Clostridium sporogenes* in processed cheese samples. The number of cells in cheese samples with 30, 40 and 50% fat in dry matter was similar during 140 days of storage.

5. CONCLUSION

The aim of the thesis was to evaluate monoacylglycerols as potential antimicrobial agents apllicable in the food industry, with a focus on monoacylglycerols of non-traditional acids. To meet the objective, the experimental work was divided into four phases. For each phase, the following conclusions can be drawn:

Phase I. Preparation of monoacylglycerols

- with optimization of reaction parameters, the addition of a particular acid to glycidol appeared to be an effective method for monoacylglycerol synthesis.

Phase II. Purification and characterization of monoacylglycerols

- recrystallization from ethanol enhanced the purity of crude recation products
- purified reaction products were obtained in a sufficient quality for antimicrobial assay
- GC-MS analysis of a new compound MAG ACA confirmed a formation of single regioisomer and NMR spectra proved the structure of asymmetrically substituted glycerol derivative

Phase III. Evaluation of antimicrobial activity of monoacylglycerols *in vitro*

- antimicrobial effect of MAGs depends on the nature of fatty acid esterified to glycerol backbone
- food-borne pathogenic and spoilage microorganisms tested were sensitive mainly to MAGs containing fatty acids with 10, 11 and 12 carbons in the chain, including MAG of unsaturated undecenoic acid
- inhibitory effects of MAGs on gram-positive bacteria can be ranked in order of growth indexes and growth indicators as follows: MAG C12:0 > MAG C11:0 > MAG C 11:1 > MAG C10:0 > MAG C18:1 > MAG ACA > MAG PFUNDA
- the most sensitive gram-positive species was *Micrococcus luteus*, *Staphylococcus aureus* was the least sensitive species
- in comparison with gram-positive species, gram-negative bacteria proved to be more resistant to MAG action with a decreasing inhibitory activity in the following order: MAG C10:0 > MAG C11:0 > MAG C11:1 > MAG C12:0 > MAG ACA > MAG PFUNDA > MAG 18:1
- in the presence of MAGs, lag-time extension, decrease in specific growth rate and decrease in cell density occurred and these changes in growth indicators were notable especially in gram-positve species
- isolated spores of *Bacillus cereus* and *Bacillus subtilis* were more sensitive to MAGs than vegetative cells of the same species
- the lowest MIC values for isolated endospores were recorded for MAG C12:0, MAG C11:0, MAG C11:1 and MAG ACA; MAG C12:0

prevented the spore germination and outgrowth even at concentration of 25 µg/ml

- antifungal activity of MAGs decreased as follows: MAG C10:0 > MAG C12:0 > MAG C11:1 > MAG ACA > MAG C11:0 > MAG PFUNDA > MAG C18:1

Phase IV: Evaluation of antimicrobial activity of monoacylglycerols in processed cheese samples

- based on *in vitro* antimicrobial activity of seven MAGs, four of them were selected and added to processed cheese samples at concentration of 0.01, 0.05 and 0.15% (w/w)
- in processed cheese samples, MAG C11:0, MAG C11:1 and MAG ACA at concentration of 0.15% prevented the growth and multiplication of both bacilli strains throughout the storage period
- clostridia were less affected by monoacylglycerols in processed cheese samples and only partial inhibition was observed in cheese supplemented with MAG C11:0, MAG C11:1 and MAG C12:0
- the most efficient monoacylglycerol in processed cheese samples was MAG ACA which caused a great reduction in microbial counts for both strains of bacilli and *Clostridium butyricum*
- the growth of bacilli 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
- no changes in appearance, smell or taste were found in processed cheese supplemented with monoacylglycerols at concentration of 0.15%

Monoacylglycerols, especially those of fatty acids with 10, 11 and 12 carbon atoms, have proven to be active in inhibition of microbial growth and multiplication *in vitro.* In addition, monoacylglycerol of undecanoic, undecenoic and adamantane-1-carboxylic acid showed the ability to prevent growth of spore-forming bacteria in processed cheese samples. Therefore, the application of these naturally occurring compounds to food products seems to be possible and promissing, as they could have beneficial effect by reducing the undesirable microbial flora and extending the shelf-life of food products.

6. CONTRIBUTION TO SCIENCE AND PRACTICE

There are numerous studies dealing with the antimicrobial effect of individual monoacylglycerols. However, studies which would compare the effects of several MAG containing fatty acids under the same conditions are still missing. One of the major contributions of the presented thesis is a comparison of inhibitory effects of seven monoglycerides on selected gram-positive and gram-negative bacteria, isolated endospores and filamentous fungi which represent pathogenic microorganisms or microorganisms that participate in food spoilage.
Several monoacylglycerols have proven to be efficient inhibitors of the growth of food-associated microorganisms and their application in foods as antimicrobial agents might be recommended. As naturally occurring compounds, monoacylglycerols hold a GRAS (generally recognised as safe) status and their record of safety allows their application to foods with great confidence.
The ability of monoacylglycerols to limit or prevent the growth of undesirable endospore forming microorganisms in real environment of processed cheese samples has also been recorded. Thus, monoacylglycerols seem to be an attractive choice since they could serve as both, antimicrobials and as an integral part of foodstuffs they could even improve functional properties of food products.

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8. LIST OF PUBLICATIONS

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