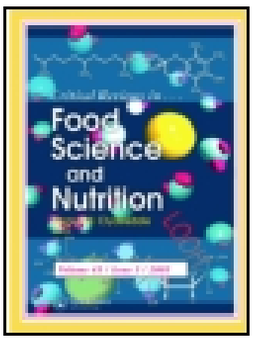

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Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

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To cite this article: Leona Buňková & František Buňka (2015): Microflora of Processed Cheese and the Factors Affecting It, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2015.1060939

To link to this article: http://dx.doi.org/10.1080/10408398.2015.1060939

Accepted author version posted online: 11 Sep 2015.

Microflora of processed cheese and the factors affecting it

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Abstract

The basic raw materials for the production of processed cheese are natural cheese which is treated by heat with the addition of emulsifying salts. From a point of view of the melting temperatures used (and the pH-value of the product), the course of processed cheese production can be considered "pasteurisation of cheese". During the melting process, the majority of vegetative forms of microorganisms, including bacteria of the family *Enterobacteriaceae*, are inactivated. The melting temperatures are not sufficient to kill the endospores, which survive the process but they are often weakened. From a microbiological point of view, the biggest contamination problem of processed cheese is caused by gram-positive spore-forming rod-shaped bacteria of the genera *Bacillus, Geobacillus* and *Clostridium*. Other factors affecting the

shelf-life and quality of processed cheese are mainly the microbiological quality of the raw materials used, strict hygienic conditions during the manufacturing process as well as the type of packaging materials and storage conditions. The quality of processed cheese is not only dependent on the ingredients used but also on other parameters such as the value of water activity of the processed cheese, its pH-value, the presence of salts and emulsifying salts and the amount of fat in the product.

Key words: processed cheese, microflora, external factors, contamination, spore-forming bacteria

1. INTRODUCTION

Properties of processed cheese

Processed cheese is cheese that has been heat-treated with the addition of emulsifying salts. Processed cheese is made in a discontinuous way by heating a mixture of natural cheeses with emulsifying salts under lower pressure and constant stirring until a homogenous mass of desired properties is formed (Carić & Kaláb, 1997; Buňka et al., 2009). During the production of processed cheese, natural cheeses which are not suitable for direct sale, i.e. cheese with different, mainly mechanical defects, can also be used (Buňka et al., 2009). However, the use of natural cheese with microbiological defects is not recommended, especially in the case of contamination by spore-forming bacteria and moulds (Glass & Doyle, 2013). Raw materials contaminated by moulds are particularly dangerous due to possible production of mycotoxins (Moss, 2002; Magan & Olsen, 2004).

The melt produced is filled into sealable containers. With regard to human health and safety of the final products, it is necessary to suppress the development of contaminating microflora. Viable microorganisms, which can be detected by conventional culture methods, should not be present in the final product or their number should be very low and thus insignificant in terms of human health. The proliferation of contaminating microorganisms can be prevented by cool storage temperature. Another possibility is autoclaving, which leads to reduction in the quantity of bacterial spores (Pflug, 1987; Glass & Doyle, 2013). However, temperatures higher than 100 °C may adversely affect the final quality of the processed cheese (Buňka et al., 2004; Lazárková et al., 2010; 2011). The shelf-life of processed cheese made in a discontinuous way (using the

melting temperatures from 90-100 °C) and stored at cool temperatures (4-8 °C) is several months (Schär & Bosset, 2002).

The aim of this paper was to describe the survival of bacteria, bacterial spores, micromycetes and yeasts in processed cheese and factors (e. g. water activity, pH, temperature) or ingredients (especially substances with emulsifying effects and fat content) that can influence the microorganisms in processed cheese.

2. MICROORGANISMS IN PROCESSED CHEESE

Microbiological changes in processed cheese depend on many factors, including the type of cheese (raw material), pH, dry matter content, sodium chloride content, concentration and type of emulsifying salts and heating temperature (Glass & Doyle, 2005). During the production of processed cheese, a heating temperature higher than 80 °C is used, which leads to killing of the vegetative forms of cells in the raw material mixture. However, these temperatures are not sufficient to kill bacterial spores (Ruf & Gläser, 1971; Glass & Doyle, 2013). The bacterial spores remain viable and, moreover, at certain pH values, the heating temperature may even stimulate their germination (Pflug, 1987). The actual values of the internal factors (mainly pH>4.5; water activity $a_w > 0.85$) also enable the bacterial spores to germinate (Loessner et al., 1997).

The microbiological quality of the final product depends mainly on the microbiological quality of the raw material used, hygienic conditions during the production as well as on the type of packaging material and storage conditions. Also, the method and conditions of the subsequent cooling of the hot wrapped melt have a significant influence on both the texture and

microorganisms. In the case of rapid cooling of the melt, the surviving microorganisms may get a "cold shock", which leads to life loss of a large proportion of the population. The effect of low temperatures is also manifested by interrupting the transport of substances through the cytoplasmic membrane. Most contaminating microflora can survive cold storage but its metabolic activity is considerably decelerated or almost reduced to minimum due to an inappropriate reaction temperature of enzymes. However, if the temperature is increased and approaches the temperature optimum of the contaminating microorganisms, their activity is restored (Jay et al., 2005; Adams & Moss, 2008).

The number of microorganisms in processed cheese may vary. Lazárková et al. (2010) found ca 4 log CFU/g of aerobic and facultative anaerobic microorganisms (total bacterial count), a similar number of aerobic and anaerobic spore-forming bacteria and approximately ten times fewer yeasts and bacteria in unsterilised processed cheese made from Dutch-type cheeses. The presence of coliform bacteria was not observed. When the processed cheese was sterilised, the microorganisms were inactivated (Lazárková et al., 2011). A similar total number of microorganisms was also observed by Palmas et al. (1999) in factory-made processed cheese spreads made from Pecorino cheese. Aly et al. (1995) detected a total number of microorganisms ca 3 log CFU/g and a similar number of spore-forming bacteria in processed cheese made from ultrafiltered retentates in laboratory conditions. The occurrence of microorganisms in processed cheese also depends on production conditions. A lower occurrence of microorganisms was found in factory-made processed cheese spreads in comparison with processed cheese made in artisanal or farmer-type dairies. A total number of microorganisms in artisanal processed cheese spreads reached more than 5 log CFU/g (Palmas et al., 1999). During storage, the number of

microorganisms in processed cheeses may increase slightly (Aly et al., 1995; Palmas et al., 1999).

The major contaminants of processed cheese include mainly psychrotrophic microorganisms or microorganisms capable of growing in the environment with low oxygen content. The most common contaminants of processed cheese include spore-forming bacteria and micromycetes. Moreover, secondary contaminants, i.e. microorganisms which enter the product after the production of the processed cheese should not be neglected (Lues & Botha, 1998; Palmas et al., 1999; ICMSF, 2005; Blackburn, 2006; Lazárková et al., 2010).

2.1. Spore-forming bacteria

As mentioned above, processed cheese and other heat-treated foodstuffs are often contaminated by spore-forming bacteria (Blackburn, 2006). Palmas et al. (1999) isolated *Bacillus cereus, B. subtilis, B. coagulans, B. pumilus, Brevibacillus laterosporus*, *Clostridum sporogenes, C. sordellii* and *C. glycolium* from processed cheese. An important feature of bacterial spores is their high resistance even to the environmental conditions which have a negative effect on vegetative forms of bacteria. Bacterial spores are resistant to the effect of higher temperatures and can also survive in an environment with a minimum amount of nutrients for a long time. Back in favourable conditions, the spores germinate from their resting form and change into vegetative cells capable of normal metabolism, growth and reproduction (ICMSF, 2005; Blackburn, 2006).

With the development of new molecular biological methods based on the analysis of the genotype of microorganisms (e.g. the comparison of 16S rRNA sequences or DNA-DNA

hybridisation), some changes in the taxonomy of microorganisms were made, which also affected the groups of spore-forming bacteria. The group of spore-forming bacteria previously included mainly the representatives of gram-positive genera *Clostridium*, *Bacillus* and *Desulfotomaculum*. As a result of the changes in taxonomy, several new genera arose from the genus *Bacillus*, including bacteria related to food contamination such as the representatives of *Geobacillus, Alicyclobacillus, Brevibacillus* and *Paenibacillus*. Some changes were also made in the taxonomy of the genus *Clostridium* and some strains of the new genera *Moorella* and *Thermoanaerobacterium* may also contribute to food spoilage (Blackburn, 2006).

Based on the growth characteristics and the relation to temperature, spore-forming bacteria can be divided into several groups:

Psychrophilic and psychrotrophic spore-forming bacteria causing spoilage of food stored at refrigeration temperatures. Important representatives are mainly anaerobic bacteria (e.g. *Clostridium estertheticum, C. gasigenes, C. putrefaciens, C. algidicarnis*) and facultatively anaerobic bacteria (e.g. *Bacillus circulans, B. mycoides, B. sphaericus*) (Boerema et al., 2002; Blackburn, 2006).

Mesophilic bacteria with an optimum growth temperature between 15 and 45 °C including (i) anaerobic proteolytic and putrefactive bacteria (e.g. *Clostridium sporogenes, C. putrefaciens*), (ii) acid-tolerant anaerobes of butyric fermentation (e.g. *C. butyricum, C. tyrobutyricum, C. pasteurianum*), (iii) facultatively anaerobic bacteria (e.g. *Bacillus circulans, Paenibacillus macerans, Brevibacillus laterosporus*) and (iv) acidophilic facultative anaerobes (e.g. *Alicyclobacillus acidoterrestris*) (Klijn et al., 1995; Blackburn, 2006). Many species from this

group were also isolated from processed cheese (Palmas et al., 1999; Lycken & Borch, 2006; Borch & Lycken, 2007).

Thermophilic bacteria with an optimum growth temperature higher than 45 °C. A negative influence of these bacteria is significant mainly in foodstuffs the production of which involves thermal heating. Facultatively thermophilic spore-forming bacteria (e.g. *Bacillus coagulans*) grow at temperatures of 55 and 37 °C. Strictly thermophilic bacteria only grow at temperatures higher than 55 °C and do not grow at 37 °C or lower temperatures. Thermophilic spore-forming bacteria include aerobic bacteria growing in non-acidic food and acidifying the environment (e.g. *B. coagulans*), thermophilic bacteria growing in non-acidic food without forming acids (e.g. *Geobacillus stearothermophilus*), thermophilic sulphur bacteria or hydrogen sulphide producing bacteria (e.g. *Desulfotomaculum nigrificans*), anaerobic bacteria not producing H_2S (e.g. *Thermoanaerobacterium thermosaccharolyticum,* previously *Clostridium thermosaccharolyticum*) and facultatively thermophilic facultatively anaerobic bacteria (e.g. *Bacillus subtilis*) (Blackburn, 2006). Within this group, e.g the strains belonging to the species of *Bacillus coagulans* were isolated from processed cheese (Palmas et al., 1999).

Bacteria of the genus *Clostridium* belong to the most common contaminants of processed cheese (Glass & Doyle, 2013). Given the fact that clostridial spores are resistant to inactivation during the manufacturing process (especially temperature under 100 °C), clostridia – mainly *C. tyrobutyricum* – may cause blowing of processed cheese (Blackburn, 2006).

Several representatives of the genus *Clostridium*, mainly *C. sporogenes, C. cochlearium* and *C. tyrobutyricum* were isolated from processed cheese showing the signs of spoilage (especially unpleasant smell) (Loessner et al., 1997; Lycken & Borch, 2006; Borch & Lycken, 2007). The

majority of cheeses from which these microorganisms were isolated were flavoured (containing meat, seafood, spices etc.) and were not stored at refrigeration temperatures (Lycken & Borch, 2006).

Processed cheese made in laboratory conditions and inoculated with *C. tyrobutyricum* did not show any visible signs of spoilage (e.g. coagulation, change in colour, gas formation). However, cheese inoculated with the species of *C. cochlearium* and *C. sporogenes* showed visible spoilage after two weeks of incubation – cavitation due to gas production, syneresis with the separation of coagulated casein and whey or change in the colour of the cheese. The gas production was lower in *C. cochlearium* compared with *C. sporogenes* (Lycken & Borch, 2006).

Pathogenic clostridia, including e.g. *C. botulinum*, can also contaminate dairy products. The spores of this bacterium are quite widespread in the environment, which increases the risk of contamination of dairy products. It was found that the minimum pH and temperature for the growth of this bacterium are pH 4.7 and 5.0 °C (Johnson et al., 1990; Glass & Doyle, 2013). Although *C. botulinum* does not belong to the most common contaminants of processed cheese, it is the focus of much attention mainly due to potential risks, abundant occurrence and danger to the human body. According to Grecz et al. (1965) the spores of *C. botulinum* preserved their viability in processed cheese stored at a temperature of 2-4 °C for four to five years, in some cheeses even longer (up to six years). At the same time, the ability to produce botulotoxin in processed cheese was observed. Between the first week and twelve months of storage at a constant temperature, the production of botulotoxin increased two to five times. After one year, the toxin content remained the same for approximately three more years followed by a decrease in toxicity. At the end of a six-year storage experiment, about half the loss of the toxin was

observed compared to the first week (Grecz et al., 1965). The production of botulotoxin is affected by many factors including pH, the concentration of sodium chloride and phosphates, dry matter content and fat in dry matter content. It was further found that the growth of *C. botulinum* and the production of toxin can be inhibited by the presence of lactic acid (Briozzo et al., 1983; Tanaka et al., 1986; Glass & Johnson, 2004a). Non-proteolytic *C. botulinum* may grow and produce toxin even at refrigeration temperatures (Graham et al., 1997). The growth of *C. botulinum* at refrigeration temperatures can also be retarded by the addition of carbon dioxide at a low concentration (Glass et al., 1999).

Apart from *C. botulinum*, other clostridial species producing botulotoxin have been described. These clostridia may be potential contaminants of processed cheese. Such representatives may include *C. butyricum*, which produces botulotoxin the type E. This bacterium was detected in connection with botulism obtained from food in Italy, China and India. The strains of *C. butyricum* were able to grow and produce toxin even at lower temperatures (12 °C) and pH (pH 4.8), e.g. in types of cheese like pesto and mascarpone. Toxin production was observed after five days of storage at 25 °C. Although this bacterium showed some properties of psychrotrophic bacteria, it was not able to grow at 4 °C (Anniballi et al., 2002).

Another possible pathogenic clostridium contaminating processed cheese is *C. perfringens*, whose probability of occurrence in processed cheese is higher than that of *C. botulinum*. This bacterium is widespread in the environment and can contaminate dairy products; the spores have been isolated from both milk and cheese. However, so far there is not enough detailed information about the diseases caused by this bacterium isolated from milk (Glass & Doyle, 2013).

2.2. Other gram-positive bacteria contaminating processed cheese

Spore-forming bacteria are not the only group involved in undesirable processes in food products. In particular, when it comes to processed cheese, non-spore-forming bacteria also cause spoilage. In many cases, it is secondary contamination when these microorganisms enter the products after the production (especially when the melt is not packed at an appropriate temperature – under 60-70 °C). These microorganisms include both pathogenic and non-pathogenic bacteria and micromycetes (Glass & Doyle, 2013; Linton & Harper, 2008). Non-spore-forming gram-positive bacteria which were isolated from processed cheese also include (i) lactobacilli (Hassanin, 1993; Lues & Botha, 1998), (ii) representatives of the genus *Micrococcus*, *Staphylococcus*, *Microbacterium* and (iii) actinomycetes (Hassanin, 1993). In isolated cases, the occurrence of *Listeria monocytogenes* (Angelidis et al., 2010; Kahraman et al., 2010) or *Staphylococcus aureus* (Palmas et al., 1999) was observed.

In the list of potential contaminants of processed cheese, gram-positive pathogens should not be neglected. One of them is, for example, a gram-positive bacterium *Staphylococcus aureus*, which is a common inhabitant of the human skin and mucous membranes and is therefore a potential secondary contaminant of many foodstuffs. *S. aureus* even tolerates higher salt concentrations and thus can grow in the environment with 15-20% NaCl (Glass & Doyle, 2013). Toxigenic staphylococci can be present in raw milk. By means of pasteurisation, the bacterial cells are inactivated but their toxin is thermostable and retains its activity even after pasteurisation. *S. aureus* is able to grow at the minimum temperature of 7 °C and pH 4.0 (Johnson et al., 1990). *S. aureus* was isolated from processed cheese spreads produced in artisanal dairies in Sardinia, Italy

(Palmas et al., 1999). Despite the fact that during the inoculation of *S. aureus* into processed cheese stored at a temperature of 30 °C for four days no significant growth was observed, the bacteria retained their viability (Glass et al., 1998). Linton & Harper (2008) stored processed cheese inoculated with *S. aureus* at 5 and 22 °C. At the lower temperature the presence of viable staphylococci was observed for 180 days, at 22 °C even for 270 days.

Another possible pathogen contaminating processed cheese is *Listeria monocytogenes* (Angelidis et al., 2010; Kahraman et al., 2010). Listeria belongs to bacteria abundantly occurring in the environment and is often isolated as a contaminant from raw milk, usually during the cold season. *L. monocytogenes* may be present even in dairies with good sanitation and hygiene. It often contaminates piping systems, surfaces or the refrigeration equipment. The minimum temperature for the growth is 1 °C and pH 4.8 (Johnson et al., 1990). In processed cheese inoculated with *L. monocytogenes* and stored at 30 °C, a decrease in the number of listeria cells was detected within four days (Glass et al., 1998). When the processed cheese inoculated with *L. monocytogenes* were stored at 5 and 22 °C, a decrease in the number of viable cells, which was the most significant within the first three days of storage, was also observed. After fourteen days, the growth of this bacterium was not observed any more (Linton & Harper, 2008). A gradual decrease in the number of viable cells of *L. monocytogenes* during storage of processed cheese at three different temperatures was also observed by Angelidis et al. (2010, 2013). The same was noted by Grassi et al. (2013) in cheese sauce for pasta stored at a temperature of 4 °C. Angelidis et al. (2010, 2013) also found that apart from the storage temperature, the kinetics of decrease in listeria in processed cheese depends on the size of the initial inoculum and differs between the individual strains applied. Within three strains tested, the clinical isolate of *L. monocytogenes*

Scott A was the most resistant. The other two strains isolated from cheese were more sensitive to the environment. When the processed cheese was stored at a temperature of 22 °C, the growth of listeria was not detected between the 40th and 50th day of storage. At a storage temperature of 12 °C, this period increased to 100-110 days and at a temperature of 4 °C up to 200-240 days (Angelidis et al., 2013). It can therefore be concluded that processed cheese belong to foodstuffs that do not support the growth of *L. monocytogenes.* However, viable cells may persist in this product for quite a long time, especially when stored at low temperatures.

In order to find the survival time, processed cheese were, apart from pathogenic bacteria, also inoculated with lactobacilli cultures. The ability of lactobacilli to survive the storage temperatures of 5 and 22 °C proved to be very good as the cells retained their viability even after 274 days of storage (Linton & Harper, 2008).

2.3. Gram-negative bacteria contaminating processed cheese

Pathogenic and potentially pathogenic gram-negative bacteria which can secondarily contaminate processed cheese include e.g. representatives of the genus *Salmonella* and pathogenic *Escherichia coli* O157:H7 or other representatives of the family *Enterobacteriaceae*. Palmas et al. (1999) isolated and identified the species *Enterobacter cloaceae, Pantoea agglomerans, Klebsiella oxytoca* and *Citrobacter freundii* from processed cheese made from Pecorino cheese (made from pasteurised sheep´s milk). *Salmonella* spp. is often detected in raw milk but its isolation from cheese is not very common. This statement is also in accord with Kahraman et al. (2010), who did not detect salmonella in processed cheese made in Turkey. The minimum values of pH and temperature for the reproduction of salmonella are pH 4.5 and 6.5 °C

(Johnson et al., 1990). A decrease in the population of *Salmonella* spp. cells in pasteurised processed cheese occurs within four days at a storage temperature of 30 °C (Glass et al., 1998). At a temperature of 5 °C, a marked decrease in the number of *Salmonella* spp. cells also occurs. After fourteen days no growth was observed. In cheese stored at 22 °C, no viable cells were observed after ten days (Linton & Harper, 2008).

The occurrence of *Escherichia coli* O157:H7 is most often mentioned in relation to meat and meat products but it can also be detected in raw milk. During the production of UHT milk it is inactivated. The minimum temperature for its growth is 2.5 °C and pH 4.6 (Johnson et al., 1990). As in the case of *Salmonella*, a decrease in the population of *E. coli* O157:H7 inoculated in pasteurised processed cheese stored at 30 °C was observed within four days. In this bacterium, the most significant decrease in viable cells was observed (Glass et al., 1998).

Other possible contaminants of processed cheese can be the bacteria causing their spoilage. Examples of such bacteria include gram-negative psychrotrophic bacteria of *Pseudomonas fluorescens* and *Aeromonas hydrophila* isolated from Italian processed cheese. The occurrence of these bacteria generally indicates secondary contamination or worse hygienic conditions during the production (Palmas et al., 1999). In the study by Linton & Harper (2008), processed cheese were inoculated with bacteria of the genus *Pseudomonas* and stored at 5 and 22 °C. Unlike the above-mentioned pathogenic species, these bacteria maintained their viability in processed cheese for a longer time. At 5 °C there was significant inactivation of the cells within the first three days and these bacteria thus behaved like pathogenic agents. However, after this period of time, the number of the cells was maintained at approximately the same level until ca the 60th day, followed by a decrease in the population. In pseudomonas, no growth was observed in

processed cheese during storage at 5 °C after 274 days but the growth was maintained even after this period of time during storage at 22 °C (Linton & Harper, 2008).

2.4. Micromycetes and yeasts

Apart from bacteria, processed cheese can also contain yeasts, yeast-like microorganisms and moulds (micromycetes). These microorganisms can cause different defects in processed cheeses (e.g. colour defects) (Daly et al., 2012). Due to the action of their lipolytic and proteolytic enzymes, they can form substances that lead to changes in the organoleptic properties of the product. Some yeasts and moulds can also grow at refrigeration temperatures in the environment with atmosphere having lower oxygen concentrations, which are properties that are ideally suited for the role of contaminants of processed cheese. Deterioration of the quality is mostly caused by the representatives of the genus *Penicillium* (*P. commune, P. roqueforti* etc.). The following yeasts – *Debaryomyces hansenii, Geotrichum candidum, Candida parapsilosis, Kluyveromyces marxianus* and moulds – *Penicillium chrysogenum, P. frequentans, P. notatum* and *P. sidowii* were isolated from processed cheese made in Italy (Palmas et al., 1999). From processed cheese made in Egypt, the moulds *Aspergillus flavus, A. niger* and *Penicillium viridicatum* were isolated. *A. flavus* was found to produce the mycotoxins B1 and B2 and *P. viridicatum* was found to produce citrinin (Hassanin, 1993). Occasionally, heat-resistant moulds, e.g. *Byssochlamys fulva* and other microorganisms characterised by resistance to increased temperatures can also be isolated from processed cheese (Blackburn, 2006).

Processed cheese in model studies were inoculated with micromycetes of the genera *Penicillium* and *Cladosporium* and then stored at temperatures of 5 and 22 °C. At a temperature of 5 °C, a gradual decrease in the viability of both micromycetes was observed. *Penicillium* sp. maintained

its viability for the whole storage period (274 days) while in *Cladosporium* sp. no growth was observed after 180 days. When the cheeses were stored at 22 °C, a decrease in the number of the cells was observed in both microorganisms during the first three days, followed by a gradual increase in the population and a more significant continual increase in the number of the cells between the 56^{th} and 274^{th} day (Linton & Harper, 2008).

Many filamentous fungi (belonging to the genera *Aspergillus, Penicillium, Cladosporium, Geotrichum* and others) have the potential to produce undesirable mycotoxins. Mycotoxins are known to be resistant to industrial processing (Sengun et al., 2008; Hymery et al., 2014). Filamentous fungi isolated from cheese- and meat-processing plants were able to produce cyclopiazonic acid, mycophenolic acid, citrinin, cladosporin, roquefortine and ergot alkaloids (Kozlovsky et al., 2014). Aflatoxin M_1 was determined in raw and UHT milk and this mycotoxin can transfer from milk to cheese (Kamkar et al., 2008; Santini et al., 2013; Cavallarin et al., 2014). Some processed cheeses are made from blue- or white-veined cheese. Various strains of *Penicillium roqueforti* are capable of mycotoxin(s) production. In many Turkish mould-ripened cheeses was detected roquefortine C, penicillic acid, mycophenolic acid and patulin (Erdogan and Sert, 2004; Cakmakci et al., 2015), *P. roqueforti* strains isolated from blue-veined cheeses in Spain were able to produce roquefortine C, PR toxin and mycophenolic acid (Fernández-Bodega et al., 2009), in blue-veined cheeses collected worldwide was detected roquefortine C and mycophenolic acid (Fontaine et al., 2015). On the other hand, in the white-veined cheeses was determined cyclopiazonic acid (Zambonin et al., 2001; Monaci et al., 2007). In other cheeses, that can be used for processed cheese production were detected various mycotoxins, e. g. sterigmatocystin (Versilovskis et al., 2009), aflatoxin M_1 (Hampikyan et al., 2010; Oliveira et al.,

2011), ochratoxin A or patulin (Pattono et al., 2013). For many details about presence of mycotoxins in cheeses see reviews of Sengun et al. (2008) and Hymery et al. (2004).

3. FACTORS AFFECTING THE MICROBIAL QUALITY OF PROCESSED CHEESE

3.1. Temperature during manufacturing

Temperature is one of the most important factors that affect the microflora in processed cheese. As mentioned above, melting temperatures used during the production of these cheeses significantly reduce the number of vegetative cells of bacteria present in the original feedstock. However, they are usually not sufficient to eliminate bacterial spores, which can cause, after germination, various defects of processed cheese. It was found that germination of the spores of *Clostridium tyrobutyricum* can be initiated, depending on the composition of the environment and other treatment procedures, at 4 °C, 30 °C or 37 °C, the most common of which was the temperature of 37 °C (Bassi et al., 2009). The storage temperature also has a significant influence on the development or slowdown in the growth of the microflora present (see the text above).

3.2. Water activity

The availability of water is one of the basic conditions for the growth and reproduction of microorganisms and can be expressed by the value of water activity (a_w). Also, the form of water binding in processed cheese is an important factor affecting the growth of microorganisms. Proper emulsification and hydration create unfavourable conditions for the growth of microorganisms (ICMSF, 2005; Jay et al., 2005), which is evident from the results of differential scanning calorimetry (DSC) as well as from the microstructure of 2 types of processed cheese

with a different fat in dry matter content (Gliguem et al., 2009). Most microorganisms involved in food spoilage require an environment with a water activity higher than 0.91. Moulds are able to grow even at a_w=0.80. Bacteria, mainly gram-negative, are relatively demanding in terms of water activity of the environment and require higher values of water activity for their growth than eucaryotic micromycetes and yeasts. Low values of water activity of the environment prolong the lag phase and reduce the growth rate and density of bacterial suspension. Microorganisms defend themselves against osmotic stress by intracellular accumulation of suitable solutes such as e.g. K^+ ions, glutamate, glutamine, proline, γ-aminobutyric acid, betaine, sucrose etc. (Jay et al., 2005).

Water activity a_w in processed cheese usually ranges between 0.91-0.96. These values of water activity are suitable to prevent the growth of some strains of *C. botulinum* and to prevent the production of their toxin. Proteolytic strains of *C. botulinum* require the values of a_w>0.935 for their growth. At lower values, the growth of these bacteria is suppressed. Similar holds true for non-proteolytic strains, for which the limiting value of water activity a_w=0.970 (ter Steeg et al., 1995). When the water activity of cheese was lower than 0.944, no production of toxin was detected while at a_w>0.957 the presence of toxin was detected. It was found that when the values of water activity range between 0.944 and 0.957, the presence of *C. botulinum* and its toxin production depend on the properties of the product, namely on the dry matter content, NaCl concentration, pH and sodium hydrogen phosphate content. The presence of ingredients that reduce the water activity and other factors of the environment such as pH, temperature and the concentration of salts and substances with antimicrobial activity thus play an important role in controlling the growth of this pathogenic bacterium (Glass & Doyle, 2013).

3.3. The pH of the environment

Most microorganisms reproduce the best at a pH around the neutral point (pH 6.6-7.5). Only a small proportion of microorganisms are able to grow at a pH below 4.0. The pH of processed cheese ranges between 5.6-6.0, which allows the growth of many microorganisms (Glass & Doyle, 2013). The inhibitory effect of low pH values is given by influencing the function of respiratory enzymes of the microbial cells and disrupting the transport of nutrients into the cell. The resistance to a pH depends on the type of the pH-lowering substance used. Monocarboxylic acids often contribute to a pH decrease in food. The effect of these acids as antimicrobial agents depends on the degree of their dissociation at a given pH (Jay et al., 2005).

The minimum pH at which *Clostridium botulinum* bacteria are able to grow is pH 4.6-4.8 for proteolytic strains and pH higher than 5.0 for non-proteolytic strains. The studies focused on the inhibitory effect of acids on *C. botulinum in vitro* showed the influence of the acids decreases in the following order: acetic acid > lactic acid > citric acid or HCl (Johnson et al., 1990). Skřivanová et al. (2005) observed the antimicrobial effects of fatty acids with 2 to 18 carbons in the chain on *Clostridium perfringens.* The results revealed that growth inhibition was achieved after the application of capric, caprylic, lauric, myristic and oleic acids. A higher inhibitory effect was achieved in the environment with a lower pH (pH 5.0-5.3) in comparison with the broth with a higher pH (pH 6.1-6.6).

The pH value of the environment also affects the spore germination of *Clostridium tyrobutyricum.* When the spores of *C. tyrobutyricum* were exposed to inductive substances of a different pH, a better effect was achieved in the case of a lower pH. In an environment with

lactic acid, germination of ca 17% of the spores occurred, with citric acid it was ca 27% and with acetic acid ca 29% of the spores of *C. tyrobutyricum* germinated (Bassi et al., 2009).

The production of botulotoxin can be prevented by the presence of some organic acids and their salts. An effective inhibitor of the formation of this toxin is e.g. lactic acid (ter Steeg et al., 1995). Sodium and potassium salts of this acid inhibit toxin production even without lowering the pH. The addition of 1.5% sodium lactate significantly decreases the production of botulotoxin in processed cheese in comparison with products without the addition of lactate (Glass & Johnson, 2004a).

3.4. The influence of substances with emulsifying effects

The most widely used agents with emulsifying effects include phosphates, polyphosphates and citrates (so called "emulsifying agents"). Apart from these substances, real emulsifiers (surfactants) such as e.g. monoglycerides can also be added to processed cheese (Buňka et al., 2009).

3.4.1. Real emulsifiers

During the production of processed cheese, monoglycerides can be used as emulsifiers. The emulsifying ability of saturated monoglycerides (MAG) depends on the type of fatty acid and increases with the growing number of carbons in the fatty acid chain (Faur, 1996; Moonen & Bas, 2004). When selected 1-monoglycerides at a concentration of 0.25% were added to model samples of processed cheese, it was found that with the growing number of carbons in the ester-linked fatty acid of MAG the rigidity of these products increases while their spreadability

decreases (Buňka et al., 2007). The number of carbon atoms and the presence of double bonds in the chain of fatty acids affect a wide variety of functional properties of these substances, and not least their antimicrobial activity (Kabara et al., 1972; Thormar & Hilmasson, 2007; Buňková et al., 2011). These substances can inhibit the growth of vegetative forms of bacteria, including the pathogenic ones (Růžička et al., 2003; Thormar & Hilmasson, 2007; Altieri et al., 2009a; Buňková et al., 2011), viruses (Thormar & Hilmasson, 2007), spore-forming bacteria (Chaibi et al., 1998; Mansour et al., 1999; Skřivanová et al., 2006) or yeasts and micromycetes (Bergsson et al., 2001; Růžička et al., 2003; Altieri et al., 2009b; Buňková et al., 2010).

Due to the lipophilic nature of fatty acids and monoglycerides, the primary goal of their attack is the cytoplasmic (biological) membrane of the cells. The mechanism of the inhibitory effect of fatty acids and their derivates on bacteria is not yet known precisely but several hypotheses have been proposed. According to some authors, they cause breakage of the biomembrane as a permeable barrier and inhibit the transport of amino acids into the cell (Dufour et al., 2007; Thormar & Hilmasson, 2007). Even micromolar concentrations can affect the activity of important enzymes in the cell membrane. Another hypothesis is based on the penetration of short- and medium-chain fatty acids into bacterial cells in an undissociated form and their dissociation within the cells, which leads to acidification of the cell content. Lower intracellular pH values disrupt the activity of intracellular enzymes and cause their inactivation (Nair et al., 2004). The antimicrobial activity of monoglycerides is affected by the composition and properties of the foodstuffs. The effects of monoglycerides can be improved by means of temperature (Dufour et al., 2007), acids (Robach et al., 1981) or chelating agents (Branen & Davidson, 2004).

However, some food ingredients (e.g. cholesterol, starch, serum albumin or phospholipids) can react with these substances and thus reduce their antimicrobial effects on contaminating microorganisms. Disadvantages of some monoglycerides, mainly those with a lower number of carbons in the ester-linked fatty acid (generally up to 12), are the changes in organoleptic properties of food caused by the presence of these substances. Their use in some foodstuffs is thus unsuitable (Davidson et al., 2005; Buňka et al., 2007; Dufour et al., 2007).

The effects of four different monoglycerides (MAG of lauric acid, undecanoic acid, undecenoic acid and adamantane-1-carboxylic acid) on the growth of *Clostridium sporogenes, C. butyricum, Bacillus cereus* and *B. subtilis* in processed cheese were observed by Hauerlandová et al. (2014). The results reveal that the MAG tested at a concentration of 0.15% (w/w) act preventively against the growth and multiplication of both species of bacilli within a 5-month storage period (6±2 °C) and thus can contribute to extending the shelf-life of processed cheese. The negative effect on clostridia was smaller. Due to the presence of MAG in processed cheese, only a partial inhibition of the growth of these bacteria was observed. The biggest inhibitory effect was observed in *C. butyricum* in processed cheese with 0.15% (w/w) MAG of adamantane-1-carboxylic acid. Similar results were also obtained when the processed cheese with the addition of monoglycerides was inoculated with the spores of the following bacteria: *B. cereus, B. subtilis, C. sporogenes* and *C. butyricum* (Hauerlandová et al., 2014).

3.4.2. Emulsifying salts

Phosphates belong to the most widely used substances with emulsifying effects during the production of processed cheese. Their effect is mainly connected with the adjustment of the

environmental conditions where they can cause a change in pH or ionic strength of the solution. In the form of sodium salts, phosphates and polyphosphates are used as emulsifying salts which ensure (usually at a concentration of 2-3%) a fine and homogenous structure of the processed cheese without the separation of water, fat and proteins (Carić et al., 1985; Buňka et al., 2009). During the production of processed cheese, phosphates have the role of emulsifying agents modifying the environment so that the caseins present could apply their emulsifying properties. This is mainly achieved by splitting off calcium from acid amino acids and phosphoseryl residues of the protein matrix and by means of peptisation, hydration and swelling of proteins or fat emulsification (Guinee et al., 2004). The cation chelation ability is also applied during the production of processed cheese, when calcium ions are attracted from caseins to phosphates by higher electrostatic forces and, on the other hand, sodium ions bind to casein (Molins, 1991; Guinee et al., 2004; Nagyová et al., 2014; Salek et al., 2015). Chelation is affected e.g. by the number of monomers in the molecule (with an increasing number of phosphates the affinity with cations rises), a particular metal cation, temperature, pH etc. Apart from the above-mentioned properties, phosphates have the ability to bind water, can affect gel formation or have an inhibitory effect on the growth of microorganisms (Molins, 1991; Buňka et al., 2009).

The antimicrobial effects of phosphates are described mainly in gram-positive bacteria, some micromycetes and yeasts (Knabel et al., 1991; Zaika & Kim, 1993; Lee et al., 1994a; Loessner et al., 1997; Suarez et al., 2005, 2007; Buňková et al., 2008; Lorencová et al., 2012). The inhibitory effect of phosphates on gram-negative bacteria are rarely described in the literature. In laboratory conditions, an inhibitory effect on *Aeromonas hydrophila* was observed (Velazquez et al., 2001).

As far as the growth inhibition of gram-positive bacteria is concerned, the inhibitory effect of phosphates is dependent on the length of their chain (condensation level). The effect of long-chain phosphates is more significant than the inhibitory effect of short-chain phosphates. Moreover, the antimicrobial effect is also influenced by temperature, pH value of the environment (higher sensitivity at pH>7.4), the initial population of microorganisms or the addition of metal ions (Jen & Shelef, 1986; Lee et al., 1994a; Zaika et al., 1997; Maier et al., 1999; Lorencová et al., 2012). Long-chain polyphosphates have a high affinity for divalent metal ions (Ca^{2+} and Mg^{2+}), which they bind readily. These ions are essential for maintaining the integrity of the cell wall of gram-positive bacteria because they form transverse bridges between the molecules of teichoic acids in the cell wall (Lee et al., 1994b). Binding of divalent ions can also be manifested by the unavailability of these ions for some essential physiological processes of the growth. Cleavage of the above-mentioned ions will result in bactericidal or bacteriolytic effects (Lee et al., 1994b; Maier et al., 1999). The ability of polyphosphates to inhibit microorganisms is weakened by the addition of polyvalent metal ions to the culture medium (Jen & Shelef, 1986; Lee et al., 1994b; Zaika et al., 1997; Maier et al., 1999).

When spore-forming bacteria are exposed to the effects of polyphosphates, the spore germination process is suppressed (Eckner et al., 1994; Loessner et al., 1997; Maier et al., 1999; Varga, 2005; Borch & Lycken, 2007). The effects of several types of polyphosphates on the growth of vegetative cells of *Clostridium perfringens* isolated from contaminated food have been examined. It was found that the desired concentrations of polyphosphates which inhibit the growth of *C. perfringens* are higher than in other bacteria. However, even the sublethal concentrations of polyphosphates reduced the sporulation ability of this bacterium (Akhtar et al.,

2008). Polyphosphates can inhibit the growth and toxin production of *C. botulinum* by metal sequestration, mainly of magnesium and calcium ions (Glass & Doyle, 2013). The application of polyphosphates on the cells of *Bacillus cereus* in the exponential growth phase led to changes in the morphology of these cells, manifested by cell lysis and the inability of septum formation during division. The sublethal concentrations of polyphosphates cause significant prolongation of the cells of this bacterium which can even form fibres (Maier et al., 1999).

Apart from observing the influence of polyphosphates on microorganisms in laboratory conditions, their effects on microorganisms in real foodstuffs have also been studied. Suarez et al. (2005) studied the inhibitory effects of commercial phosphates on micromycetes isolated from food plants and concluded that the sensitivity of micromycetes to phosphates is species specific and highly dependent on the length of the polyphosphate chain. Molins et al. (1985) dealt with the presence of *Clostridium sporogenes* in stored meat products. They found that the addition of phosphates can significantly decrease the number of these bacteria.

It has also been found that polyphosphates are effective inhibitory agents against microorganisms involved in the spoilage of milk products, mainly cheese spreads. The addition of polyphosphates to these products can slow down or prevent the growth of undesirable spore-forming bacteria (Briozzo et al., 1983; Eckner et al., 1994; Loessner et al., 1997; Varga, 2005; Borch & Lycken, 2007). Polyphosphate salts have an inhibitory effect against the originator of the late blowing of cheese, i.e. *C. tyrobutyricum.* The growth and gas production of this bacterium in processed cheese were reduced by adding 0.5-1.0% of polyphosphates (Loessner et al., 1997).

Potassium salts were suggested as an alternative to reduce the amount of sodium salts in processed cheese. However, their effects against *C. botulinum* were not reliably proved (Karahadian et al., 1985). Regardless of the ionic strength, the inhibitory effect of sodium hydrogen phosphate against *C. botulinum* is better than the effect of potassium hydrogen phosphate. Sodium salts in processed cheese with a reduced fat content (5% fat) suppress the production of botulotoxin in *C. botulinum*. However, potassium salts exhibit only a 75% anti-botulinum activity in comparison with sodium salts (Glass & Johnson, 2004a).

Apart from phosphate emulsifying salts, citrate-based emulsifying salts are also used during the production of processed cheese. However, if we observe the inhibitory effects of citrates, mainly on microorganisms involved in the spoilage of processed cheese due to gas production, the antimicrobial effect of the addition of polyphosphates and monophosphates is better in comparison with citrates (Glass & Doyle, 2013). Many other studies also show smaller inhibitory effects of sodium citrate against the growth of *C. botulinum* compared to phosphates (Karahadian, et al., 1985; ter Steeg et al., 1995).

3.5. Fat content

Some studies suggest that the fat content in the raw materials can also affect the growth of undesirable microorganisms in processed cheese. It was found that the growth of anaerobic bacteria was reduced in the environment of processed cheese with a lower fat content in comparison with products (with the same content of dry matter, salts and pH) in which the fat content was not reduced (ter Steeg et al., 1995).

The environment with a reduced fat content appears to be less favourable for many other bacteria as well, e.g. *Listeria monocytogenes* and *Salmonella* sp. The growth of these bacteria was inhibited more rapidly in low fat cheese in comparison with cheese without a reduced fat content. Also, many fatty acids such as capric, lauric, oleic and linoleic acids have inhibitory effects against the above-mentioned microorganisms in cheese (Glass & Doyle, 2013).

Similar results were also obtained by Hauerlandová et al. (2014), who studied the behaviour of spore-forming bacteria of the genus *Bacillus* (*B. cereus* and *B. subtilis*) and *Clostridium* (*C. butyricum* and *C. sporogenes*) in processed cheese with a fat in dry matter content of 30, 40 and 50% within a ca 140-day storage period at a refrigeration temperature. The results of the study show that the growth of bacilli in cheese with a different fat content was not really affected by the fat content although the highest number of cells was found in processed cheese with the highest fat content. In the clostridia observed, the results were similar with the exception that in cheeses containing 50% of fat in dry matter, a significantly better growth of clostridia was observed in comparison with cheese with a lower fat content. At the same time, no significant differences in the number of microorganisms were observed in cheese with a different fat content inoculated with vegetative cells and spores of these species of bacilli and clostridia (Hauerlandová et al., 2014).

A decrease in the growth of selected clostridia was observed in the presence of free fatty acids (mainly caprylic, capric, lauric, myristic, oleic and linoleic acids) in a medium with a fat content of 20% (Glass & Johnson, 2004a,b). Lauric acid showed the best inhibitory effects against *Clostridium* spp. (Glass & Johnson, 2004b, Skřivanová et al., 2006).

Lipids seem to form a protective environment for bacteria and protect them against antimicrobial agents soluble in the aqueous phase. They can thus reduce the inhibitory effect of some antimicrobially active substances, e.g sorbic acid, potassium sorbate, monolaurin and polyphosphates (Glass & Johnson, 2004a,b). Another explanation of this mechanism may lie in the interaction of lipophilic parts of antimicrobial agents with lipid molecules, which may result in a change of the inhibitory effect (McLay et al., 2002).

Also, a fat content may affect the production of botulotoxin. In cheese with a low fat content and practically fat-free cheese, the production of this toxin may be delayed in comparison with full-fat cheese. In processed cheese with a fat in dry matter content of <1% or <5%, the production of this toxin was not observed within 56 weeks of storage at 27 °C while botulotoxin was detected in similar cheese with 20% of fat in dry matter after four weeks of storage at the same temperature. A statistical analysis showed that a fat content and raw material composition have a significant influence on the delay of botulotoxin production in processed cheese and similar products (Glass & Johnson, 2004a; Glass & Doyle, 2013).

3.6. The influence of other factors and ingredients

The growth of spore-forming bacteria, e.g. *Bacillus subtilis,* at a pH of 6.0 can also be inhibited by propionic acid. At a lower pH (4.0-5.0) propionic acid also has an inhibitory effect on yeasts and micromycetes. Its salts can also be considered to be antimicrobially active substances (Davidson et al., 2005). The inhibitory effects of sodium propionate on *Listeria monocytogenes, Staphylococcus aureus, Lactobacillus plantarum, Proteus vulgaris, Torula* sp. (Davidson et al., 2005) or that of calcium propionate on *Bacillus subtilis* and *L. monocytogenes* (Janes et al., 2002;

Davidson et al., 2005) are known from the literature. Although the effective concentrations of propionic acid and its salts differ somewhat in the individual publications, generally, the concentrations that stop the growth of microorganisms range between 0.1-1.0%.

Sorbic acid is able to slow down or prevent the growth of bacteria, yeasts or micromycetes (Razavi-Rohani & Griffiths, 1999). Its effect on microorganisms contaminating food is given, as in the case of lactic acid, by the ability to dissociate intracellularly. However, its use is restricted to foodstuffs with a high content of lipids due to the fact that the solubility of sorbic acid in water is low as it dissolves much better in fats. Sorbates, mainly potassium sorbate, are used in cases where higher solubility in water is desired. Therefore they are often used in foodstuffs with a high water content. An indisputable advantage of sorbic acid and its salts is the fact that these substances do not affect the taste or smell of the products to which they are added (Davidson et al., 2005). Potassium sorbate is considered to be non-toxic for humans. Its inhibitory effects have been demonstrated on a broad spectrum of bacteria, mesophilic and psychrotrophic bacteria, bacteria of the family *Enterobacteriaceae*, facultatively anaerobic bacteria and lactobacilli (Mendonca et al., 2006). Sorbic acid can be added to processed cheese in an amount up to 0.2% of the weight of the final product. Some studies report that the addition of potassium sorbate at a concentration of 0.13-0.26% reduces the growth of *C. botulinum* and toxin production (Glass & Doyle, 2013).

Also, nisin, for example, which is produced e.g. by many strains of *Lactococcus lactis*, has inhibitory effects, mainly against gram-positive bacteria. Nisin is soluble in water, it binds to phospholipids of the cytoplasmic membrane and thus distorts its permeability. As in the case of many other antimicrobial agents, spores are much more resistant to the effects of nisin than

vegetative cells. According to some studies, the use of nisin leads to a reduction in spore germination (Delves-Broughton et al., 1996). The addition of nisin (4 000-10 000 U nisin/g) can also decrease the production of botulinum toxin in processed cheese (Somers & Taylor, 1987). Cheese spreads made from cheddar with the addition of nisin-producing lactococci inoculated with *Clostridium sporogenes* showed a significantly longer shelf life in comparison with control samples without nisin (Zottola et al., 1994). The addition of nisin is permitted in many countries. In the USA it is permitted at a concentration of 250 mg/kg (Glass & Doyle, 2013), in EU at a concentration of 12.5 mg/kg (Regulation (EC) No. 1333/2008).

The growth of bacteria and toxin production can also be slowed down by the addition of lysozyme, which is commonly found in milk, eggs and mucosal secretions. This enzyme degrades the cell wall of bacteria and is more effective against gram-positive bacteria in comparison with gram-negative bacteria (Glass & Doyle, 2013).

Also, cinnamon essential oils or their components show antifungal effects. In the conditions *in vitro* as well as on the surface of cheese it was found that cinnamon leaf essential oil and cinnamon bark essential oil inhibit the growth of *Penicillium* spp. and *Cladosporium* sp., which were isolated as cheese contaminants (Jeong et al., 2014). Balaguer et al. (2013) incorporated cinnamaldehyde into a gliadin film which was applied as active antifungal food packaging on bread and on the surface of cheese spreads. In the conditions *in vitro*, the gliadin film incorporating cinnamaldehyde inhibited the growth of *Penicillium expansum* and *Aspergillus niger*. When the film containing 5% of cinnamaldehyde (5 g cinnamaldehyde/100 g protein) was applied, the effect on both moulds was considered to be fungicidal. Subsequently, this antifungal film was applied on the inner side of the lid which was part of the packaging of cheese spread

stored at 4 °C. The comparison with the control samples (on which this active film was not applied) revealed that active food packaging containing a gliadin film incorporating cinnamaldehyde at a concentration of 5% inhibits both naturally occurring moulds and *P. expansum*, which the cheese was inoculated with. In the case of untreated cheese spreads stored at 4 °C, the growth of moulds was observed after ca. 14 days. When the cheese spreads were actively packed in the gliadin film incorporating cinnamaldehyde, the growth of moulds was observed after ca 30 days of storage.

4. CONCLUSION

Microflora of processed cheese is primarily affected by the production method of this type of cheese. Also, many external and internal factors may influence the qualitative and quantitative representation of microorganisms in processed cheese. The typical attribute of processed cheese spoilage is gas formation (due to the hydrogen or carbon dioxide production) and off-odors caused by production of microbial unfavourable substances (e. g. organic acids, such as butyric or acetic acid). The factors that significantly affect microbial colonisation and spoilage of this type of cheese include cheese variety, pH, water activity (moisture), heat treatment, fat content, NaCl content, the addition of substances with emulsifying effects or the addition of other food additives.

ACKNOWLEDGEMENT

This work was supported by The National Agency for Agriculture Research, project No. QJ1210300, The Complex Sustainable Systems programme.

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