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Short communication

Effects of copper on germination, growth and sporulation of *Clostridium tyrobutyricum*

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ABSTRACT

The effects of copper (Cu^{2+}) on spore germination, vegetative growth and sporulation of *Clostridium tyrobutyricum*, which is capable to causing texture and flavour defects in Emmental cheese, were studied. Spore suspensions of three different strains were used as starting material for two experimental set-ups. The first studied the effects of supplemented (0–30 ppm) copper in RCM medium during spore germination and vegetative growth of *C. tyrobutyricum* measured by plating. The second set-up studied the effects of copper (0–30 ppm) in RCM medium during growth and sporulation of *C. tyrobutyricum* as measured by optical density at 550 nm and by platings after heat treatment of the samples respectively. Inhibition of germination, vegetative growth and sporulation processes by copper was strain-dependent. Both sporulation and germination were more sensitive than vegetative growth of *C. tyrobutyricum* to the inhibitory effects of copper. Copper, at the concentrations investigated in this study, inhibits spore germination of *C. tyrobutyricum* strains. Consequently copper may reduce the risk of late blowing spoilage from in the germination of *C. tyrobutyricum* spores during the ripening period of Emmental cheese.

1. Introduction

Traditional Swiss Emmental cheese manufacture is based on the use of copper (Cu) cheese vats. Production capacities of modern plants for Emmental cheese manufacture are much larger than traditional scale; for both economical and hygiene reasons, copper cheese vats have been replaced by stainless steel vats. When copper vessels are used, "tinning" of copper and brass vessels protects from excessive contamination by copper, yet it is a procedure often neglected because of cost and effort (IPCS, 1998). In Finland, the standard Emmental cheese manufacture practice includes a supplement of copper as $CuSO_4$ salt in cheese milk in order to reach the same copper concentration as cheese milk in the copper vat would have. In many countries, Emmental cheese is manufactured in stainless steel vats without any copper salt supplement. This is the case for organic Emmental cheese manufacture in Finland, because of the organic food regulations. There are only few food groups, maximal Cu contents of which have been regulated, but these do not include cheeses. The overall effects of copper on the final quality of Emmental cheese are unknown and controversial. In the manufacture of traditional Emmental cheese, copper has been suggested to regulate an important part of the ripening process by influencing growth of starters and the proteolytic activity of the participating microorganisms; under- or overdosing of copper has been claimed to cause quality defects mainly by inhibition of propionic acid fermentation (Sieber et al., 2006). On the other hand, Dolezalek et al. (1979) reported that increased Cu content in cheese milk did not affect the growth of starters, but had a negative effect on the ripening and quality of Emmental cheese, and consequently they concluded that the use of stainless steel vats is advantageous.

Butyric acid fermentation caused by spore germination and followed by vegetative growth of *Clostridium*, mostly *Clostridium tyrobutyricum*, is one of the major causes of spoilage in semi-hard and hard ripened cheeses including Emmental. Also known as late blowing, this spoilage by *Clostridium* produces both texture and flavour defects during the ripening period of Emmental cheese (Fox et al., 2000; Aureli and Franciosa, 2003). Pasteurisation treatment of cheese milk is not enough to eliminate the risk of clostridial spoilage because *Clostridium* spores in raw milk easily resist standard pasteurisation temperatures. Various heat treatments to which cheese milk and cheese curd are subjected to during cheese manufacturing can activate in fact spore germination, which in turn promotes for clostridial growth and late blowing defects during

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cheese ripening (Foster and Johnstone, 1990). Very few microbial physiological data are available on those conditions and factors in ripening the Emmental cheese matrix which promote or prevent clostridial spore germination and vegetative growth.

Copper contents of cheese milk and Emmental cheese vary because of different manufacture practices employed. Copper is one of the external factors which could influence the risk of clostridial spores germination and/or vegetative growth, and consequently the quality of Emmental cheese. Published data on this subject turned out to be very limited despite the economical importance of late blowing defects in Emmental and other semi-hard and hard ripened cheeses. In order to investigate the importance of copper in this context, the effects of various concentrations of supplemented Cu in growth medium on germination, growth, and sporulation processes of various *C. tyrobutyricum* strains have been studied.

2. Materials and methods

2.1. Preparation of spore stock culture

The following three *C. tyrobutyricum* strains were considered in this study: The strain ATCC 25755 from the American Type Culture Collection, the strain DSMZ 664 from German Collection of Microorganisms and Cell Cultures, and the strain VHB 8 from Valio Ltd. R&D unit (Helsinki, Finland). The strain VHB 8 has been isolated from a late blowing spoiled Emmental cheese; phenotypic and genotypic characterization classified this strain as *C. tyrobutyricum*. In addition, this strain has been found to be effective in sporulation (Dr. Soile Tynkkynen from Valio Ltd., personal communication). The strains were cultivated in RCM broth (composition in g/L: meat extract 10, peptones 5.0, yeast extract 3, D (+) glucose 5, starch 1, NaCl 5, sodium acetate 3, L-cysteine chloride 0.5, and agar–agar 1) tubes or on the RCM agar plates anaerobically at 37 °C for 72 h. Anaerobic conditions were created by using BBL (Baltimore Biological Laboratories) gas pack anaerobic system (Becton Dickinson & Co., Cokceysville, Mich., U.S.A.). The strains were stored in RCM broth together with 20% glycerol at −80 °C for long term storage. For each strain a fresh RCM culture was used for the series of experiments.

Each strain of *C. tyrobutyricum* was cultivated on RCM agar plates at 37 °C under anaerobic conditions for 72 h. For the preparation of the spore stock culture of each strain, a few colonies from the plate were harvested with a sterile loop and resuspended in 1 ml 0.85% NaCl solution in a 1.5 ml eppendorf test tube, and the cell suspension was heat-treated at 80 °C for 10 min to kill vegetative *C. tyrobutyricum* cells (Bergère and Sivelä, 1990).

2.2. Effects of copper supplement on spore germination, vegetative growth and sporulation

In this study, two experimental set-ups were designed: The germination–vegetative growth (G–V) study, and the vegetative growth–sporulation (V–S) study. In both cases the starting material was a fresh spore stock culture of the strain. Copper supplements (7.5, 15 or 30 ppm Cu) were added in sterile RCM broth (Merck) or sterilised, warm RCM agar (Merk). Cu stock solution, from which the different Cu concentrations were adjusted in the RCM broth/melted RCM agar, was freshly prepared as $CuSO_4$ solution in sterile water (corresponding Cu content of 2550 ppm), and filter-sterilised (pore size 0.45 μm, Schleicher & Schuell, Dassel, Germany). RCM medium without copper supplementation was used as the control. In the G–V study a proper 10-fold dilution series of the spore stock culture was made in sterile 0.85% NaCl solution, and 50 μl sample from each dilution was spread on an RCM agar plate containing 0 (control), 7.5, 15 or 30 ppm Cu. One set of plates was incubated as indicated above for 48 h and the other set for 72 h, and the numbers of colonies were counted. The experiment was carried out in duplicate. In the V–S study 1.0% (v/v) of the spore stock preparation was added to the RCM broth (150 ml final volume) and incubated under anaerobic conditions at 37 °C for 24 h to complete spore germination. After 24 h incubation the RCM culture was divided into 2.5 ml subcultures and supplemented with 0 (control), 7.5, 15 or 30 ppm Cu using freshly prepared filter-sterilized $CuSO_4$ stock solution (2550 ppm Cu). One set of subcultures was further incubated under anaerobic conditions at 37 °C for 24 h and the other set of subcultures was incubated under the same conditions for 48 h. From each subculture, the growth of *C. tyrobutyricum* was measured spectrophotometrically at 550 nm (Novaspec II spectrophoto-meter, Amersham Pharmacia, Sweden) using a non-inoculated RCM broth containing the corresponding Cu concentration and having same incubation conditions for each blank, in order to correct any possible colour variation caused by the presence of Cu in the medium. Heat-resistant spores were measured by plating on RCM agar plates as described above after heat-treating the sample at 80 °C for 10 min. These experiments were carried out in triplicate.

2.3. Statistical analyses

Statistical evaluation was performed by two-way analysis of variance (ANOVA) with Bonferroni's post-test to compare mean using PRISM (4·0), GraphPad Inc. (San Diego CA, U.S.A.). Differences were considered to be significant at $P < 0.05$.

3. Results and discussion

In order to reveal effects of copper (Cu) on *C. tyrobutyricum* three processes were considered in this study: spore germination, growth of vegetative cell, and sporulation of vegetative cell. Each of these processes may be expected to include a specific, copper-sensitive step(s) or reaction(s), which could be the principal factor explaining the observed total effect of copper on a particular strain. In practice, these processes are very difficult to separate from each other, since they are part of the life cycle of sporulating bacteria. The experimental plan of this study is based on two set-ups. In the first experimental set-up it is possible to reveal the total effect of copper during spore germination and vegetative growth processes of a particular *C. tyrobutyricum* strain, and in the second experimental set-up, the total effect of copper during vegetative growth and sporulation processes of a particular *C. tyrobutyricum* strain. In order to reveal strain-dependent variation of copper effects, three strains (ATCC 25755, DSMZ 664 and VHB 8) of *C. tyrobutyricum* species were included in this study.

The study concerning the effects of copper on the spore germination and vegetative growth of *C. tyrobutyricum* strains revealed a strain-dependent difference in sensitivity to total effect of copper (Fig. 1). The strain ATCC 25755 (Fig. 1) was the most resistant, for it could reach practically the same level of CFU ml^{-1} in the presence of 7.5 ppm Cu as in the absence of copper (0 ppm Cu), contrary to the strains DSMZ 664 and VHB 8 (Fig. 1), which began to be irreversibly affected in the presence of 7.5 ppm Cu. In the presence of higher Cu content (15 and 30 ppm), the three strains did not differ: all three strains were irreversibly and completely inhibited in the presence of 15 ppm Cu (statistically significant ($P < 0.05$) redutions in 15 ppm Cu columns in Fig. 1) and 30 ppm Cu, respectively (zero levels in 30 ppm Cu columns in Fig. 1). Copper could effect during spore germination or vegetative growth process or during both. Longer cultivation time (72 h instead of 48 h) did not show any significant recovery effect (Fig. 1). Successful spore germination process is a prerequisite for colony formation (vegetative growth). Dose-dependent, irreversible