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Effect of modified atmosphere and temperature abuse on the growth from spores and cereulide production of *Bacillus weihenstephanensis* in a cooked chilled meat sausage

Line Thorsen [a,*], Birgitte Bjørn Budde [a], Anette Granly Koch [b], Trine Danø Klingberg [a]

[a] Department of Food Science, Faculty of Life Science, Copenhagen University, Frederiksberg C, Denmark
[b] Danish Meat Research Institute, Roskilde, Denmark

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ABSTRACT

The effect of modified atmosphere packaging (MAP) on the germination and growth of toxin producing psychrotolerant *Bacillus* spp is not well described. A model agar system mimicking a cooked meat product was used in initial experiments. Incubation at refrigeration temperature of 8 °C for 5 weeks of 26 *Bacillus weihenstephanensis* including two emetic toxin (cereulide) producing strains showed that *B. weihenstephanensis* is sensitive to MAP containing CO_2. The sensitivity to 20% CO_2 was dependent on strain and oxygen level, being increased when oxygen was excluded from the MAP. Growth from spores was observed at the earliest within 2 weeks when 20% CO_2 was combined with 2% O_2 and in 3 weeks when combined with "0"% O_2 (the remaining atmosphere was made up from N_2). Results were validated in a cooked meat sausage model for two non-emetic and one emetic *B. weihenstephanensis* strain. The packaging film oxygen transfer rates (OTR) were 1.3 and 40 ml/m^2/24 h and the atmospheres were 2% O_2/20% CO_2 and "0"% O_2/20% CO_2. Oxygen availability had a large impact on the growth from spores in the MAP meat sausage, only the most oxygen restricted condition (OTR of 1.3 ml/m^2/24 h and "0"% O_2/20 % CO_2) inhibited growth of the three strains during 4 weeks storage at 8 °C. Cereulide production was undetectable during storage at 8 °C irrespective of choice of the MAP (quantified by liquid chromatography mass spectrometry/mass spectrometry). MAP storage at 8 °C for 1 and 3 weeks followed by opening of packages and temperature abuse for 1.5 h daily at 20 °C during 1 week resulted in increased cell counts and variable cereulide production in the meat sausage. A pre-history at 8 °C for 1 week in MAP with OTR of 1.3 or 40 ml/m^2/24 h and 2% O_2 resulted in cereulide concentrations of 0.816–1.353 µg/g meat sausage, while a pre-history under the most oxygen restricted condition (OTR of 1.3 ml/m^2/24 h, "0"% O_2/20 % CO_2) resulted in minimal cereulide production (0.004 µg/g meat sausage) at abuse condition. Extension of MAP storage at 8 °C for 3 weeks followed by abuse resulted in a substantially reduced cereulide production.
Data demonstrates that MAP can be used to inhibit growth of a psychrotolerant toxin producing *Bacillus* spp. during chill storage at 8 °C, and substantially reduce the risk of emetic food poisoning at abuse condition. Results are of relevance for improving safety of ready to eat processed chilled foods of extended durability.

1. Introduction

Bacillus cereus is a spore forming Gram positive bacterium with the potential to cause diarrhoeal and emetic food poisoning (Kramer and Gilbert, 1989) through the production of enterotoxins (Michelet et al., 2006) and the heat stable toxin cereulide (Shinagawa et al., 1995), respectively. While it has been known for some time, that the closely related psychrotolerant species *B. weihenstephanensis* also produces enterotoxins (Pruss et al., 1999; Stenfors et al., 2002), it was not until recently that its ability to produce cereulide was identified (Thorsen et al., 2006). Strains of psychrotolerant *B. cereus* able to grow at 7 °C have been related to several diarrhoeal food poisoning incidents (Carlin et al., 2006). In comparison, the role of psychrotolerant *B. weihenstephanensis* in diarrhoeal and emetic food poisoning is unknown. Due to its recent discovery only few environmental conditions affecting its cereulide production have been examined (Thorsen et al., 2006; Thorsen et al., submitted for publication), and its potential for causing emetic intoxications therefore needs further investigation.

Ready to eat foods, including processed chilled foods of extended durability are increasingly being produced and consumed in Europe. The frequent occurrence of *B. cereus* in these products (Rosenquist et al., 2005; Wijnands et al., 2006) constitutes a potential health risk (Ehling-Schulz et al., 2004; Jääskeläinen et al., 2004). Especially the psychrotolerant spore formers including *B. cereus* and *B. weihenstephanensis* frequently isolated from chilled foods such as milk, meats and their products (Lechner et al., 1998; Wijnands et al., 2006) are considered a potential health risk, because the spores can survive a cooking process and germinate and grow at refrigeration temperatures of 4–7 °C (Borch and Arinder, 2002).

While several foods including meat dishes and meat products are associated to *B. cereus* diarrhoeal food poisoning, it is mostly

* Corresponding author. Tel.: +45 35 33 33 26; fax: +45 35 33 32 14.
E-mail address: lith@life.ku.dk (L. Thorsen).

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farinaceous foods served at restaurants and in private homes, which are associated to emetic food poisonings (Kramer and Gilbert, 1989). Recently, however cereulide production has been detected in industrially produced foods such as cheese and bacon (Rajkovic et al., 2007), which are kept for extended time at refrigeration temperatures. It has furthermore been suggested that starch in combination with protein rich substrates such as meat and eggs, may enhance cereulide production (Jääskeläinen et al., 2004). Knowledge of cereulide production in foods including these ingredients and which are stored for extended time at refrigeration temperature needs to be investigated.

B. cereus is tolerant to levels of salt (NaCl) and pH (Martinez et al., 2007) of cooked meat products (e.g. pH 5.8–6, 1.5–2% (w/w) NaCl), and additional preservation techniques are needed to prevent germination and growth. Modified atmosphere packaging (MAP) is used by the food industry to increase shelf life of a large variety of chilled foods (Daniels et al., 1985). Studies showing that *B. cereus* is sensitive to carbon dioxide (CO_2), which is used as an active gas in MAP, have primarily been demonstrated at non refrigeration temperatures of ≥10 °C (Enfors and Molin, 1978; Sutherland et al., 1996). Carbon dioxide is suggested to inhibit *B. cereus* spore germination by increasing cell membrane fluidity, thereby disturbing activity of membrane bound enzymes essential to spore germination (Enfors and Molin, 1978). The vegetative growth is suggested to be inhibited by the CO_2 penetrating the bacterial membrane resulting in acidification of the internal pH and interference with metabolic processes and certain enzyme systems (Daniels et al., 1985). Few studies investigating the effect of CO_2 discriminate between mesophilic and psychrotolerant strains of *B. cereus* (Bennik et al., 1995; Sutherland et al., 1996; Werner and Hotchkiss, 2002) and the effect of CO_2 on germination and growth of *B. weihenstephanensis* and the effect on cereulide production of emetic *B. weihenstephanensis* have not been reported previously.

Another important risk factor is that temperature abuse of MAP food products often occurs during transportation and during storage in domestic refrigerators increasing the risk of food poisoning (Nauta et al., 2003; Notermans et al., 1997). Furthermore once packages are opened, the protective effect of MAP disappears. Data on the effect of temperature abuse and opening of MAP packages on *B. cereus* and *B. weihenstephanensis* germination, growth and toxin formation is necessary to estimate the risk of diarrhoeal and emetic food poisoning.

Domestic refrigerator temperatures are often higher than the recommended temperature of 5 °C (Nauta et al., 2003; Notermans et al., 1997). The aim of this study is to examine the effects of MAP on growth of *B. weihenstephanensis* and psychrotolerant *B. cereus* strains from spores during incubation at a slightly abusive temperature of 8 °C. For this purpose a model agar system and a cooked meat sausage model were used. To examine critical consumer behaviour, some of the packages were opened and subjected to temperature abuse at 20 °C. In addition, the effect of the mentioned conditions on emetic toxin formation of *B. weihenstephanensis* MC118 (Thorsen et al., 2006) was examined. The outcome of this study will be of relevance to improve safety of ready to eat chilled foods of extended durability.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used include 50 *B. weihenstephanensis* strains (no. 1-48, MC67 and MC118) and 4 psychrotolerant strains of *Bacillus cereus* (no. 49-52) all isolated from the soil of a sandy loam on Møn, Denmark (Hendriksen et al., 2006; Thorsen et al., 2006). These strains were kindly provided by the National Environmental Research Institute, Roskilde, Denmark. A further 3 *B. weihenstephanensis* strains of food origin are included. These are, *B. weihenstephanensis* strain 453-92 isolated from cream (Stenfors and Granum, 2001), *B. weihenstephanensis* INRA 161 (Guinebretiere and Nguyen-the, 2003) from raw courgette and *B. weihenstephanensis* DSM11821 isolated from milk (Lechner et al., 1998). The three strains of food origin were provided by P. E. Granum, The Norwegian School of Veterinary Science, Oslo, Norway, the Institut National de Recherche Agronomique, Avignon, France and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany, respectively. All strains were maintained at −40 °C in 20% (v/v) glycerol (Merck, Damstadt, Germany).

2.2. Preparation of spores

Frozen cultures were propagated aerobically in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, England) for 18 h at 30 °C. A volume of 0.1 ml was spread on T3-agar (Travers et al., 1987) and stored for 6 days at 30 °C. Spores were verified by phase contrast microscopy, harvested and resuspended two times in cold (5 °C) saline containing 0.5% (w/v) or 2% (w/v) NaCl. The spore concentration was adjusted to 7 $\log_{10}$ /ml using a hemocytometer and kept at 5 °C until use, for a maximum of 4 days.

2.3. Effect of atmosphere on the growth from spores on BHI-agar

Effect of atmosphere on the growth from spores of selected *B. weihenstephanensis* and *B. cereus* strains was examined on the surface of BHI-agar. The BHI-agar plates were inoculated with 100 µl heat activated spore suspension (30 min at 75 °C), were stored at 8 °C, and examined for visible growth once a week during 5 weeks. Initially all of the 57 strains including 53 *B. weihenstephanensis* strains and 4 *B. cereus* strains were screened for anaerobic growth (<1% O_2) on BHI-agar plates pH 7.0, 0.5% (w/v) NaCl. Packaging of plates was in 65 µm thick low-density-polyethylene (LDPE) stomacher bag (Bie & Berntsen, Rødovre, Denmark) with an anaerobic generator (Anaerogen, Oxoid) and indicator (Oxoid) as well as wet tissue paper moistened with sterile distilled water to avoid desiccation. Following these experiments 26 of the 57 strains were selected based on the different tolerances to anaerobic atmosphere ranging from very tolerant with growth within 2 weeks (12 strains), 3 weeks (5 strains), 4 weeks (1 strain) to non tolerant with no growth in 5 weeks (8 strains). The 26 strains were examined for visible growth on BHI-agar plates, with pH adjusted to 6 and with a NaCl concentration of 2% (w/v). Packaging was in NEP 120 bags (oxygen transfer rate (OTR): 1.3 ml/m^2 and CO_2 TR: approximately 5.2 ml/m^2 both measured at 24 h, 23 °C, 1 atm., 50% relative humidity) from AMCOR FLEXIBLES (Horsens, Denmark) by use of a Multivac A 300/52 gas packaging unit (Multivac, Germany). Four different atmospheres were applied; a) atmospheric atmosphere, b) 0% O_2/20% CO_2/80% N_2, c) 2% O_2/20% CO_2/78% N_2 and d) 0% O_2/60% CO_2/40% N_2 with gas supplied by Air Liquide (Ballerup, Denmark). If the head space varied more than 5% from the average, as determined by Archimedes law, re-packaging was performed. Gas concentrations of packages were analyzed using a CheckMate 9900 CO_2/O_2 gas analyzer (PBI Dansensor, Denmark) immediately after packaging and before opening of packages showing visible growth. Gas composition and pH were measured for non inoculated plates at the beginning and end of the storage period of 5 weeks. The pH was measured using a PHM 93 (Radiometer, Copenhagen, Denmark).

2.4. Effect of atmosphere and packaging material on growth and cereulide production by B. weihenstephanensis in a meat model

The effect of two different atmosphere combinations and two different packaging materials on the growth from spores and cereulide production of three *B. weihenstephanensis* strains MC118 (emetic), strain 37 (non-emetic) and strain INRA 161 (non-emetic) were examined. The three strains had been selected based on their different tolerances to growth in 0% O_2/20% CO_2/80% N_2, i.e. strain