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Control of Shigatoxin-producing *Escherichia coli* in cheese by dairy bacterial strains

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ABSTRACT

Bio-preservation could be a valuable way to control Shigatoxin-producing *Escherichia coli* (STEC) in cheese. To this end, 41 strains were screened for their inhibitory potential on model cheese curd and on pasteurized and raw milk uncooked pressed cheeses. Strains of Lactococcus lactis, Lactococcus garvieae, Leuconostoc pseudomesenteroides, Leuconostoc citreum, Lactobacillus sp, Carnobacterium mobile, Enterococcus faecalis, Enterococcus faecium, Macrococcus caseolyticus and *Hafnia alvei* reduced STEC O26:H11 counts by 1.4–2.5 log cfu g^{-1} and to a lesser extent STEC O157:H7 counts in pasteurized milk cheeses. Some strains can act in synergy to inhibit STEC in raw milk uncooked pressed cheeses. Inhibitory associations had no adverse effect on the sensory characteristics of these cheeses. The association of *H. alvei, Lactobacillus plantarum* and *Lc. lactis* was the most inhibitory: after inoculation of this consortium into milk, STEC O26:H11 and O157:H7, inoculated at 2 log cfu ml^{-1}, were reduced by up to 3 log cfu g^{-1} in ripened cheese. Inhibition in cheese cannot be predicted from H_2O_2 production in BHI medium, decreased pH or milk reduction. It is not clear what role the rapid decrease in pH during the first 6 h may play in the inhibition. Further studies will be needed to determine the nature of the inhibition.

1. Introduction

Shiga-Toxin-producing *Escherichia coli* (STEC) can cause a wide range of clinical symptoms including non-bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and death (Center for Deseases Control and Prevention, 2000; Meng et al., 2007). Raw milk cheeses are rarely responsible of food outbreaks. One *E. coli* O157:H7 outbreak was associated with consumption of raw milk Gouda cheese (Honish et al., 2005) and another with consumption of fresh unpasteurized goats' milk cheese (Espié et al., 2006a). One *E. coli* O26:H11 outbreak was associated with unpasteurized cow's milk cheese (Espié et al., 2006b).

E. coli O157:H7 and especially O26:H11 can grow and survive to varying degrees during cheese manufacture and ripening, depending on the cheese-making method applied (D'Amico et al., 2010; Farrokh et al., 2013; Miszczycha, 2013; Reitsma and Henning, 1996; Vernozy-Rozand et al., 2005). It is difficult to have a general overview of STEC contamination in cheese as measurement methods have varied from one country to another. Contamination was estimated at 0.9% in France (Bulletin épidémiologique, santé animale et alimentation n°55) and 0.6% in Germany (EFSA, 2010). The infective dose of STEC is less than 10 bacteria in the case of susceptible groups (Schmid-Hempel and Frank, 2007) so, to ensure cheese safety, no STEC must be present. It is therefore very important to control STEC not only during milk production but also throughout the cheese process. To this end, the bio-preservation by using selected LAB strains has been described as an interesting approach to controlling the development of undesired or pathogenic microorganisms and further improve the quality and safety of cheese (Grattepanche et al., 2008; Favero et al., 2015). But only a few bacterial species are able to inhibit STEC in cheese. A strain of *Hafnia alvei* was able to reduce counts of a strain of *E. coli* O26:H11 by 0.8–1.4 log cfu g^{-1} in a pasteurized milk uncooked pressed cheese, by unknown mechanisms (Delbès-Paus et al., 2013). *In vitro*, STEC have been inhibited by bacteriocins such as nisin (Murdock et al., 2006), colicin (Lyon and Olson, 1997) and antagonistic compounds such as reuterin (El-Ziney and Debevere, 1998). Haberbeck et al. (2015) state that the growth limiting pH for 188 STEC strains ranged between 3.8 and 4.3 with 0 mM of lactic acid and between 4.3 and 4.8 with 25 mM of

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lactic acid in Luria Bertani medium. *E. coli* count was reduced by 2 log cfu ml^{-1} in the presence of hydrogen peroxide at a concentration of 0.7 mM in a laboratory medium (Watts et al., 2003).

The aim of this study was to evaluate the growth of Shigatoxin-producing *E. coli* (STEC O26:H11 and O157:H7) in uncooked pressed cheeses after inoculating antagonistic strains (principally lactic acid bacteria) into milk. First, strains with antagonist properties *in vitro* were tested for their inhibitory activity in model curd, and for their ability to produce H_2O_2 in a laboratory medium, to decrease pH and to reduce the redox potential of milk. Subsequently, the effect on STEC O26:H11 and O157:H7 growth of individual strains, alone or in combination, was evaluated in pasteurized and raw milk uncooked pressed cheeses. The most strongly antagonistic strains were also tested for their impact on the sensory qualities of raw milk uncooked pressed cheeses.

2. Materials and methods

2.1. Screening of the inhibitory power of bacterial strains in model cheeses

2.1.1. Bacterial strains and culture conditions

Forty-one bacterial strains antagonistic to *Listeria*, STEC and *Staphylococcus aureus* were selected from the URF (Unité de Recherches Fromagères, INRA, Aurillac, France) and VetAgro Sup collections (Table 1). They were cultivated and counted on M17, Man Rogosa Sharpe MRS and Brain heart Infusion BHI and inoculated after overnight conservation at 4 °C.

Two strains of STEC serotype O26:H11 (strains 21765 and F43368) and one of O157:H7 (strain FCH6), isolated from milk and cheese, were provided by VetAgro Sup, Marcy l'Etoile, France. They were cultivated 24 h in buffered peptone water before their inoculation.

2.1.2. Sensitivity of STEC strains to H_2O_2

The three STEC strains (O26:H11 strains 21765, F43368 and O157:H7 strain FCH6) were inoculated at 10^8 cfu ml^{-1} (in order to better detect their sensibility to the H_2O_2) into BHI broth in the presence of H_2O_2 at 0.25, 0.5, 0.75, 1, 1.5 and 2 mM and incubated for 18 h at 42 °C. They were then enumerated on Violet Red Bile Glucose (VRBG) agar at 42 °C for 24 h.

2.1.3. Production of hydrogen peroxide (H_2O_2) by bacterial strains

Bacterial strains were inoculated into BHI broth at 10^7 cfu ml^{-1}, incubated at 30 °C for 24 h and agitated at 200 rpm for 3 h before sampling. Hundred µl of culture, centrifuged for 2 min at 12,500 rpm, were used to fill wells cut into plates of Prussian blue agar (Saito et al., 2007). Positive controls were performed with 100 µl of different dilutions of H_2O_2 in sterile water (0.3 mM, 0.6 mM, 1.1 mM, and 1.6 mM). The quantity of H_2O_2 produced by the strains was estimated by measuring the radius of clear zone around the well in comparison to the positive controls.

2.1.4. pH and redox potential measurement in milk

Sterilized reconstituted milk (120 g l^{-1} milk powder) was inoculated at 10^6 cfu ml^{-1} and cultures were carried out for 24 h in water baths at temperatures simulating the decrease in temperature recorded in uncooked pressed cheese, as described by Alomar et al., 2008. The variations in pH and redox potential (Eh) in milk were recorded continuously, using the CINAC system (Corrieu et al., 1988). The minimum Eh value (Eh min) was determined (Brasca et al., 2007).

2.1.5. Preparation of model cheese curd

The control model cheese curd was prepared with 50 ml of pasteurized milk incubated at 33 °C and inoculated with *Streptococcus thermophilus* at 10^6 cfu ml^{-1} and STEC strain 21765 at 10^2 cfu ml^{-1}. For the assay cheese curds, each bacterial strain (Table 1) was also added at 10^6 cfu ml^{-1}. The milk was coagulated by 20 µl of calf rennet (Beaugel Ets Coquard, Villefranche sur Saône, France) for 35 min at 33 °C and then centrifuged for 20 min at 14,000 rpm, 33 °C. The supernatant was discarded and the pellet was incubated for 24 h in a temperature-controlled batch reactor programmed to mimic the decrease in temperature during the manufacture of uncooked pressed cheeses (decreasing from 33 °C to 24 °C over 24 h) (Alomar et al., 2008). Each strain was tested 2 or 3 times.

2.1.6. Preparation of small pasteurized and raw milk uncooked pressed cheeses

Small (500 g) uncooked pressed cheeses were made with 5 l of raw or pasteurized milk from an agricultural school farm (ENILV, Aurillac, France). For the controls, the milk was inoculated only with *St. thermophilus* at 10^6 cfu ml^{-1} and the STEC strain at 10^2 cfu ml^{-1}. For the assay cheeses, bacterial strains, singly or in combination (Table 2), were also added to the milk at 10^6 cfu ml^{-1}. The inoculated milks were processed by the uncooked pressed cheese method described by Callon et al. (2011). Twenty-four hours after curding, the cheeses were dried at 9 °C, 70% relative humidity (RH) and 50% ventilation for 4 h and then ripened at 9 °C in sterile stainless steel boxes in a cellar for 8 or 28 days. After 8 and 18 days of ripening, the cheeses were washed with sterile salt water (20% NaCl).

2.1.7. Preparation of Saint-Nectaire type cheeses for challenge tests

Saint-Nectaire type cheeses (1.7 kg) were manufactured with 40 l of raw milk from ENILV prepared with 0.6% of a commercial starter culture (MY800, *St. thermophilus*, *Lactobacillus delbrueckii* spp. *bulgaricus*, Danisco, Paris La Défense, France) and a commercial mould culture of *Penicillium commune* (2.5 ml 200 l^{-1}) (Laboratoire Interprofessionnel de Production -LIP, Aurillac, France). Raw milk was inoculated with STEC O26:H11 (strain 21765) or O157:H7 (strain FCH6) at 10^2 or 0.05 cfu ml^{-1}. In some assays, a consortium composed of *Lactobacillus plantarum* (FH3), *Lactococcus lactis* (D5.3) and *H. alvei* (B16) was added at 10^6 cfu ml^{-1}. Cheeses were manufactured as described in 2.1.6. They were washed and ripened for 28 days on stainless steel shelves in a cellar at 9 °C, 96% relative humidity and 5% ventilation. The experiment was performed twice.

2.1.8. Preparation of Saint-Nectaire type cheese (1.7 kg) for sensory analysis

Two hundred and 40 l of raw milk from ENILV were divided into six batches. Forty liters were only inoculated with commercial starter MY800 and commercial mould as described in 2.1.7. Each of the five other batches was inoculated with the commercial strains and also with either *H. alvei* (B16) alone or one of the following anti-STEC associations: (1) *H. alvei* (B16) + *Lc. lactis* (N718); (2) *H. alvei* (B16) + *Lc. lactis* (D5.3); (3) *H. alvei* (B16) + *Lb. plantarum* (FH3) + *Lc. lactis* (D5.3); (4) *H. alvei* (B16) + *M. caseolyticus* (RP8). Saint-Nectaire type cheeses were manufactured and ripened as described in 2.1.7.

2.2. Analyses

2.2.1. Microbial analysis

Pasteurized samples (25 ml of milk or 5 g of curd or 10 g of cheese core) were taken from the milk at 0, 2, 3 and 6 h after the strains had been inoculated, from model curd at 24 and 48 h and from small uncooked pressed cheeses at 6 h, 24 h, 48 h and 8 d. Enumeration of the STEC strains was performed on VRBG medium