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Production of polyclonal antibodies against spores of *Clostridium tyrobutyricum*, a contaminant affecting the quality of cheese: characterisation of the immunodominant protein

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The effect of different treatments in the production of polyclonal antibodies against *Clostridium tyrobutyricum* spores and its immunodetection was studied. Moreover, the main protein that causes the immunological response has been characterised. Antisera from rabbits immunised with non-treated, heated or sonicated spores were tested by ELISA. Heated and sonicated spores gave a faster increase in antibody titre than non-treated spores a long time after immunisation. Heat treatment improved reactivity when it was applied to the spores prior to immunodetection. However, when the highest titre was reached, the treatment effect was not observed either in the immune response or in the immunodetection. The immunodominant protein was identified in a spore extract by transfer-blotting, analysed by on-line liquid chromatography tandem mass spectrometry, and characterised by comparison of its peptide fragmentation spectrum with the NCBI database. Five proteins from the family of chaperonins were shown related to the immunodominant protein of *C. tyrobutyricum* spore extract.

**Keywords:** polyclonal antibodies; *Clostridium tyrobutyricum*; spore antigens; heat treatment; sonication

**Introduction**

During the last 20 years, butyric contamination of milk has been an important problem causing severe economic loss to the cheese industry. The butyric defect is characterised by a disorganisation of the cheese internal structure and by organoleptic alterations, such as unpleasant smell and taste (McSweeney & Fox, 2004). This defect is caused by the presence in milk of spores of *Clostridium tyrobutyricum*, an anaerobe Gram-positive bacterium. This microorganism is considered the main agent responsible for the late spoilage in brine-salted semi-hard and hard cheeses, e.g. Emmental, Gouda or Edam cheeses (Berge`re & Lenoir, 2000). This anaerobe bacterium is present in the soil, develops in silage, and contaminates milk during milking (Bergère & Sivelä, 1990).

Milk containing spores of *C. tyrobutyricum* can be consumed as liquid milk or used in manufacturing most dairy products without any problem. However, it is necessary to screen milk for the microorganism when used to produce mature cheese, so that if milk is contaminated it can be used for other purposes to avoid future
problems. Consequently, the ‘ideal’ method to detect this bacterium should be fast (<24 h), specific and extremely sensitive because even when C. tyrobutyricum is present in very low numbers in milk (i.e. 500 spores/l), it could cause serious defects in cheese maturation (Talbot, Robreau, Gueguen, & Malcoste, 1994).

Most methods for detecting spores of C. tyrobutyricum are based on spore germination and vegetative cells growth. The commonly used most-probable-number (MPN) method is only semi-quantitative, it takes up to 7 days to yield results, and their specificity is often uncertain (Bergère & Sivelâ, 1990). Methods such as a direct immunodetection assay for C. tyrobutyricum spores after membrane filtration of milk (Bourgeois, Leparc, Abgrall, & Cleret, 1984; Neddellec, Cleret, Robreau, Talbot, & Malcoste, 1992) or those using specific DNA probes (Herman, De Block, & Van Renterghem, 1997; Klijn, Nieuwenhof, Hollwerf, Vanderwaals, & Weerkamp, 1995; López-Enríquez, Rodríguez-Lázaro, & Hernández, 2007) have been described previously.

The immunomagnetic separation (IMS) methods allow the specific recovery of target bacteria from highly heterogeneous suspensions (Safarik, Safarikova, & Forsythe, 1995). IMS methods have been used successfully for detection of different bacterial species on several types of food matrix (Blake & Weimer, 1997; Muramatsu, Maruyama, Yanase, Ueno, & Morita, 1996). These methods rely upon the interaction between surface antigens of target cells and specific antibodies that are attached to paramagnetic beads (Olsvik et al., 1994). The first step in the development of an IMS method to isolate and concentrate C. tyrobutyricum from milk is to obtain specific antibodies directed against the spore surface antigens.

The aim of this work was to study the effect of heat and ultrasound treatments applied to C. tyrobutyricum spores, as a way to improve the production of polyclonal specific antibodies. We also identified and characterised the protein fraction of the spore that causes the immunogenic response.

Methods

Culture and sporulation of C. tyrobutyricum

The C. tyrobutyricum strain used in this work was isolated from a late blowing cheese and donated by ZEU-Inmunotec (Zaragoza, Spain). A volume of 40 ml from a 20-h grown C. tyrobutyricum culture in Reinforced Clostridium Medium (RCM; Scharlau, Barcelona, Spain) was inoculated into cellulose dialysis membrane of 12–14,000 cut-off (Medicell, London, UK) immersed into triptone-glucose-yeast extract (TGE) sporulation medium (Berge & Hermier, 1970; Cerf, Bergère, & Hermier, 1967). The device to culture C. tyrobutyricum was composed of a large glass tube closed by a three-holed rubber stopper (Schneider, Grecz, & Anellis, 1963). The three holes were fitted with three different tubes, each with different functions: one to inoculate the dialysis membrane with the culture, another to create anaerobic conditions, and the third hole allowed the gas release from the culture during cellular growth. For the last function, N₂ was bubbled through the tube into the TGE medium for 5 min before and 10 min after culture inoculation. The culture was incubated at 37°C for at least 72 h. The suspension obtained was then centrifuged at 4000 × g for 15 min at 4°C, and washed three times with sterile distilled water.
Obtaining *C. tyrobutyricum* spores

Non-sporulated cells were lysed by incubation with a 400 μg/ml solution of lysozyme (Sigma, St. Louis, MO, USA) at 42–45°C overnight. Spores of *C. tyrobutyricum* were separated from vegetative cells debris in a Percoll® gradient (Sigma), as previously described (Leuschner, Weaver, & Lillford, 1999). On top of the gradient, 2 ml of the spore suspension was gently added. Separation was carried out by centrifugation at 4000 × g for 30 min at 4°C. Spores were present as a firm pellet in pure Percoll® (density 1.13) at the bottom of the centrifuge tube, whereas debris and remaining vegetative cells were distributed within the lower density layers. The presence of >95% refringent spores was confirmed using phase contrast microscopy. Spores were harvested, washed four times with sterile distilled water, and finally resuspended in sterile distilled water at a concentration of 10⁸ spores/ml and stored at −20°C until used.

Spore sonication and heating

A 0.5 ml aliquot of a 10⁸ spores/ml suspension was sonicated at 50 kHz for 15 min in an ultrasonic bath. Aliquots of 0.5 ml of a spore suspension of the same concentration were heated in a water bath at 75°C for 15 min.

Preparation of antisera

Polyclonal antisera raised against spores of *C. tyrobutyricum* were obtained in our laboratory by immunisation of female White New Zealand rabbits as follows. Four rabbits were immunised with different types of spores; two animals with heat-treated spores, one rabbit with sonicated spores, and one with non-treated spores. Animals were injected in multiple subcutaneous injections with 0.5 ml of a 10⁹ spores/ml suspension in 0.15 M NaCl, 10 mM K₂HPO⁴/KH₂PO⁴ buffer, pH 7.4, mixed with the same volume of Freund’s complete adjuvant (Sigma) as primary dose, followed by monthly boosts of the same dose in Freund’s incomplete adjuvant (Sigma). Rabbits were bled from the marginal vein 15 days after booster injections. Blood was allowed to coagulate spontaneously at room temperature, and serum was separated from coagulum by centrifugation at 500 × g for 15 min, pooled and stored at −20°C until used. Presence of specific antibodies against *C. tyrobutyricum* was checked by immunodotting.

Immunodotting assay

A 2 μl volume of *C. tyrobutyricum* spore suspension (10⁸ spores/ml in distilled water) was spotted on a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). When samples were dried, the membrane was incubated in 0.14 M NaCl, 3 mM KCl, 2 mM KH₂PO₄, 8 mM Na₂HPO₄ buffer, pH 7.4 (PBS) containing 5% skimmed milk powder for 1 h at room temperature to block non-specific binding of proteins. After five washes with PBS, the membrane was incubated for 1 h at room temperature with the antisera of immunised rabbits diluted 1:100 in PBS containing 1% skimmed milk powder. As negative control, a piece of membrane treated in the same way was incubated with the pre-immune serum of the corresponding rabbit. Membranes were washed as previously described and then incubated with
HRP-conjugated goat anti-rabbit IgG antibody (Sigma) diluted 1:2000 in PBS with 1% skimmed milk powder. After five washes with PBS, the membranes were revealed with a solution of 3 mg 4-cloro-1-naftol (Merck, Darmstadt, Germany) in 1 ml methanol, 2 ml distilled water and 5 μl hydrogen peroxide. The reaction was stopped by soaking the nitrocellulose membranes in distilled water.

**Enzyme-linked immunosorbent assay (ELISA)**

Different microorganism suspensions were used to coat 96-wells Maxisorp microtitration plates (Nunc, Roskilde, Denmark). The samples were diluted to 10^6 cell or spore/ml in 50 mM Na₂CO₃/NaHCO₃ buffer, pH 9.6, and a volume of 100 μl of this suspension was added to each well and incubated overnight at 4°C. Some wells were coated with Na₂CO₃/NaHCO₃ buffer without spores as a blank. The plates were washed four times with PBS and 0.05% Tween-20 (PBST), and then blocked with 5% skimmed milk powder in PBS for 2 h at room temperature. Polyclonal rabbit antisera were diluted with PBS containing 1% skimmed milk powder in consecutive 2-fold dilutions from an initial 1/8 dilution. The corresponding dilution (100 μl) was added to the appropriate well and left to incubate for 1 h at room temperature. Each dilution of antisera was tested in duplicate. The plates were washed as previously described, and 100 μl of a 1:2000 diluted anti-rabbit IgG-HP conjugate (Sigma) in PBS with 1% skimmed milk powder was added to each well. The plates were incubated for 1 h at room temperature and then washed four times with PBST. Finally, 100 μl of tetra-methyl-bencidine substrate (TMB; ZEU-Inmunotec, Zaragoza, Spain) was added to each well, and plates were left to incubate for 10 min while colour developed. A volume of 100 μl of a 2 M H₂SO₄ solution was added in order to stop the enzymatic reaction, and absorbance was measured at 450 nm.

**Preparation of spore extract**

Proteins from *C. tyrobutyricum* spore were extracted following the method described by Tyrell, Bulla and Davidson (1981). Four 1 ml samples at 10^8 spores/ml were centrifuged and each pellet was resuspended in 1 ml of 6 M guanidine hydrochloride and 0.1 M β-mercaptoethanol at pH 8.6 and incubated at room temperature for 20 min. The disrupted spores were subsequently dialysed in a cellulose membrane with a molecular weight cut-off of 6000–8000 against 0.1 M Tris–HCl and 9 M urea, pH 8.6, for 24 h, and then against distilled water for 48 h. Finally, the spore extract was lyophilised.

**Polyacrylamide gel electrophoresis and transfer blotting**

Lyophilised spore extract (2 mg) was solubilised by incubation in 1 ml of 1% SDS (w/v), 1% β-mercaptoethanol (v/v) and 6 M urea in 0.01 M NaH₂PO₄–Na₂HPO₄ buffer, pH 7.2, for 1 h at room temperature. The solubilised extract and molecular weight standards (Pharmacia Amersham, Uppsala, Sweden) were treated in 2.5% SDS buffer at 100°C for 5 min and then subjected to SDS electrophoresis using 8–25% polyacrylamide gradient gel (Pharmacia Amersham). Electrophoresis was carried out on a Phast-System unit, according to the manufacturer’s instructions.
As the samples were run in duplicate, after electrophoresis the gel was cut, and one half was stained for 1–2 h in 0.065% Coomassie blue R (w/v) in methanol:acetic acid:glycerol:distilled water at the proportion 30:10:10:50 (v/v/v/v), and destained for 2–4 h in methanol:acetic acid:glycerol:distilled water at the proportion 25:8:2:65 (v/v/v/v). The second half of the gel was used for transfer blotting carried out in a Milliblot-SDE system (Millipore, Billerica, MA, USA) for 15 min at 1–1.2 mA/cm² of gel. The immunological detection of antigens transferred to nitrocellulose was performed by immunodotting assay as described above.

**Immunogenic protein characterisation**

The band corresponding to the protein that reacted with the rabbit antiserum against *C. tyrobutyricum* spores was carefully cut from the Coomassie stained gel. The piece of gel was transferred to an Eppendorf vial with 50 µl of destaining solution. The sample was analysed by the Proteomic Unit of Barcelona Scientific Park (Barcelona, Spain).

The procedure is briefly summarised in the following steps: proteins were reduced and alkylated by treatment with a 10-mM dithiothreitol solution and a 55-mM iodine acetamide solution, respectively. Afterwards, the proteins were in-gel digested overnight at 37°C with 0.27 nM trypsin (sequencing grade modified). Tryptic peptides were extracted from the gel matrix with 10% formic acid and CH₃CN, and dried in a vacuum centrifuge. The extracts were resuspended in 10 µl of 10% formic acid solution and analysed by on-line liquid chromatography tandem mass spectrometry. Data of the eluted peptides were generated in PKL file format and submitted for database searching in MASCOT server (Perkins, Pappin, Creasy, & Cottrell, 1999). The search parameters were as follows: maximum of one missed cleavage by trypsin, charged state of +2 and +3, and mass tolerance of ±0.25 Da. Taking into account these parameters and searching in the Eubacteria Database Probability Based Mowse Score, protein scores higher than 47 indicate identity or extensive homology (p <0.05).

**Results**

*Production of antiserum anti-*C. tyrobutyricum*

Rabbit antisera analysed by the immunodotting technique gave positive reaction against *C. tyrobutyricum* spores, while the corresponding pre-immune sera did not give any reaction. The specificity of antiserum was checked against two different strains of *C. tyrobutyricum* (CECT 4011 and CECT 4012), *C. sporogenes* (CECT 892), *C. butyricum* (CECT 361), *C. acetobutylicum* (CECT 508), *Lactobacillus plantarum* (CECT 220) and *Geobacillus stearothermophilus* (CECT 4516) by immunodotting assay. These seven microorganisms listed were obtained from the ‘Spanish Type Culture Collection’ (CECT, Valencia, Spain). All the antiserum recognised the *C. tyrobutyricum* strains, but also reacted against *C. sporogenes* and *C. butyricum*. *C. acetobutylicum* only produced a weak signal, and *L. plantarum* and *G. stearothermophilus* did not react with the antiserum. To estimate the magnitude of cross-reactivity of the antiserum against *C. tyrobutyricum* spores with the rest of the microorganisms, non-competitive ELISA was performed and the results are shown in Figure 1. At low dilutions of antiserum, the reactivity was similar for
C. tyrobutyricum, C. sporogenes and C. butyricum. At dilutions above $2^{-10}$, we found a higher reactivity against C. tyrobutyricum spores used for immunisation (ZEU-Inmunotec provided the strain). Vegetative cells of C. tyrobutyricum from both CECT 4011 and CECT 4012 strains gave more reaction than the spore-form of the CECT 4011 strain, although the antisera were obtained by immunisation with spores. We also observed the same reactivity for C. tyrobutyricum cells and C. sporogenes, intermediate reactivity for C. acetobutylicum and C. butyricum, and very low reactivity for L. plantarum and G. stearothermophilus. At dilution $2^{-14}$, C. acetobutylicum, C. butyricum, L. plantarum and G. stearothermophilus gave undetectable reaction, while the signal from C. tyrobutyricum remained high.

Antisera titres were determined using a non-competitive ELISA assay, as described in the Methods section, and defined as the reciprocal of the dilution at which half saturation was achieved in the titration curves. Serum samples from the five first bleedings of the four rabbits were analysed by ELISA against the non-treated C. tyrobutyricum spores. The titration curves shown in Figure 2 correspond
to the antisera of the five bleedings obtained from the rabbit immunised with non-treated spores. The pattern obtained for the antisera from other rabbits along immunisations was very similar to Figure 2. As the graph shows, the titre increases from the first to the second bleeding, from a titre of 91 to 362, and there is a small increase between the third and fourth bleedings. However, between the fourth and fifth injections, a delay of 10 weeks was applied and the titre markedly increased to 46,341. In subsequent experiments, similar to those carried out for the identification of the immunodominant protein (described below), antisera with the highest titre were used.

**Effect of treatments of C. tyrobutyricum spores on antibody production**

To study the effect that different treatments applied to *C. tyrobutyricum* spores before their inoculation in rabbits had on their immune response, antisera were tested by non-competitive ELISA using plates coated with non-treated spores. The values of antiserum titres from the five bleedings are shown in Table 1. In the first three bleedings, we observed that the antiserum obtained from the rabbit immunised with non-treated spores did not reach as high a titre compared to those shown by antisera.
from rabbits immunised with heated or sonicated spores. In the second bleeding, the titre of the antisera from rabbits immunised with treated spores was between 1024 and 5793, antiserum from the rabbit immunised with non-treated spores only showed a titre of 362 and did not reach the same titre as the other antisera until the fourth bleeding. The differences between the three types of immunisation practically disappeared at the fifth bleeding, when the highest titres were reached for all the antisera.

**Effect of treatments on *C. tyrobutyricum* spores on recognition by antibodies**

In order to study the effect of the spore treatment prior to immunodetection, immunoreactivity of the antisera against non-treated, sonicated or heated spores was

<table>
<thead>
<tr>
<th>Bleeding</th>
<th>Rabbit 1 (non-treated spores)</th>
<th>Rabbit 2 (sonicated spores)</th>
<th>Rabbit 3 (heated spores)</th>
<th>Rabbit 4 (heated spores)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>91</td>
<td>64</td>
<td>2896</td>
<td>64</td>
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<td>2</td>
<td>362</td>
<td>2024</td>
<td>5793</td>
<td>1024</td>
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<td>3</td>
<td>1024</td>
<td>4096</td>
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<td>4096</td>
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<td>4096</td>
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<td>4096</td>
<td>2896</td>
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<tr>
<td>5</td>
<td>46,341</td>
<td>65,536</td>
<td>65,536</td>
<td>65,536</td>
</tr>
</tbody>
</table>

Figure 3. Differences in the immunodetection of non-treated (■), heated (▲), and sonicated (○) spores by antisera from the first (A) and the fifth (B) bleedings. Each point in the curves corresponds to the mean value of the absorbance obtained by ELISA using the four rabbit antisera analysed by duplicate in two independent experiments. Error bars correspond to standard deviation.
also checked by non-competitive ELISA (results are shown in Figure 3). In the first bleeding (Figure 3A), differences were observed only between immunoreactivity against non-treated or sonicated spores and heated spores. However, in consecutive bleedings, no significant improvement was found in the immunodetection, even when spores were treated by heat, as shown in the graph of results for the fifth bleed (Figure 3B). No differences were observed in the reaction between antisera and non-treated or sonicated spores for any of the bleedings.

**Identification of the immunodominant spore protein**

Proteins of *Clostridium* spores are insoluble and require denaturing and reducing agents to render them soluble (Wiencek, Klapes, & Foegeding, 1990). The extraction of *C. tyrobutyricum* spore wall proteins was carried out with 6 M guanidine hydrochloride and 0.1 M β-mercaptoethanol, pH 8.6. This extraction buffer had been used previously for extraction of spore coat proteins from *Bacillus* species (Tyrell et al., 1981) and was found effective in our work to extract *C. tyrobutyricum* spore proteins.

When the solubilised spore extract of *C. tyrobutyricum* was subjected to SDS-PAGE, several bands comprised of a wide range of molecular weight were detected after Coomassie staining (Figure 4A). The separated spore proteins were transferred from the gel to nitrocellulose and analysed by blotting, using a pool of positive rabbit

![Figure 4](image_url)

**Figure 4.** (A) Electrophoresis pattern of *C. tyrobutyricum* spore proteins solubilised in 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 6 M Urea, 0.01 M NaH₂PO₄:Na₂HPO₄, pH 8.6 (lane 3). Low molecular weight standards (lane 1) were phosphorylase B (97,000), albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400). High molecular weight standards (lane 2) were myosin (212,000), α2-macroglobulin (170,000), β-galactosidase (116,000), transferrin (76,000) and glutamic dehydrogenase (53,000). (B) Transfer-blotting of the protein extract of *Clostridium tyrobutyricum* spores. Comparison with the electrophoretic pattern of standards indicates that the immunogenic protein corresponds to a band located between 66 and 76 kD.
antisera from the fifth bleeding. The results in Figure 4(B) show that antibodies reacted only with one band of the spore extract proteins. The relative molecular weight of the immunoreactive band was estimated measuring the migration distance of the band in the electrophoresis gel and interpolating this value in the calibration plot made with the relative migration distance of the LMW and HMW standards. The band reacting with antisera against spore proteins of *C. tyrobutyricum* showed an apparent molecular weight of 73 kD.

However, the analysis by transfer-blotting of proteins from *C. tyrobutyricum* spores separated by electrophoresis, using the antisera from the first, second and fifth bleedings, revealed differences in the immunoreactive proteins along the time (Figure 5). While in the first bleeding, antibodies recognised a high molecular weight band corresponding to an apparent molecular of 126 kD (Figure 5A), in the subsequent bleedings, this protein was weakly recognised and the protein band corresponding to 73 kD appeared to be the most immunoreactive. This protein seems to be the immunodominant antigen, since it has also been recognised in our laboratory by antisera produced in sheep (data not shown).

Characterisation of the immunodominant spore protein

Proteins associated with the electrophoretic band of 73 kD, which reacted with antisera against *C. tyrobutyricum* spores, were analysed by on-line liquid chromatography tandem mass spectrometry. The comparison of the peptides fragmentation spectrum obtained from the tryptic digestion of the sample with the National Center for Biotechnology Information (NCBI) database resulted in the identification of five significant proteins (Table 2). The identified proteins belong to microorganisms from the order of Clostridiales, but the sequence coverage (percentage of the peptides of the identified protein present in the sample) in all cases is <10%. In addition, all the
identified proteins are chaperonins, protein complexes from the cytosol or mitochondria which form an important class of heat shock proteins that prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress, into their native and functional state.

**Discussion**

In this work, by immunising rabbits with the entire spores of *C. tyrobutyricum*, we obtained polyclonal antibodies that recognised the two tested strains of this species. Polyclonal antibodies against *C. tyrobutyricum* have also been obtained by other authors (Bergère, 1985; Scolari et al., 1993) who also reported cross-reactions with the spores of closely related species, such as *C. sporogenes* or *C. butyricum*. This was also the case for *C. sporogenes* in the current work (Figure 1). Furthermore, Scolari et al. (1993) also obtained results of antiserum titres lower for spores than for vegetative cells in agreement with our results. As the aim of our work was to study the immunogenicity of *C. tyrobutyricum* spores, the cross-reactions have not been considered a problem. In addition, the cited species are also responsible for late blowing in cheese, thus in some cases, their detection could be advantageous. However, in further studies, as the antibodies are intended for the specific detection of *C. tyrobutyricum*, immunoadsorption of antisera with related species should be performed in order to eliminate cross-reactions.

The pattern observed in the titre values of the obtained antisera is in accordance with a normal immune response in which the second antigen injection results in an increase in the antibody titre that usually remains high after subsequent booster injections. The high increase in the concentration of antibodies that we have found after a long delay between two boosts has also been previously described (Deshpande, 1996).

Table 2. Proteins identified from the electrophoretic band recognised by antisera against *C. tyrobutyricum* spores using on-line liquid chromatography tandem mass spectrometry (MALDI-TOF).

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Theoretical mass (Da)</th>
<th>Mowse score</th>
<th>Coverage (%)</th>
<th>No. of matching peptides</th>
<th>Protein identification and species</th>
</tr>
</thead>
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<tr>
<td>gi 25452841</td>
<td>58,297</td>
<td>184</td>
<td>7</td>
<td>4</td>
<td>60 kDa chaperonin (Protein Cpn60) <em>(Clostridium botulinum)</em></td>
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<tr>
<td>gi 149091590</td>
<td>66,124</td>
<td>121</td>
<td>4</td>
<td>3</td>
<td>DnaK5 <em>(Clostridium kluyveri)</em></td>
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<tr>
<td>gi 15894564</td>
<td>65,723</td>
<td>99</td>
<td>4</td>
<td>3</td>
<td>Molecular chaperone DnaK <em>(Clostridium acetobutylicum)</em></td>
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<tr>
<td>gi 38327296</td>
<td>48,805</td>
<td>74</td>
<td>5</td>
<td>2</td>
<td>GroEL <em>(Ruminococcus albus)</em></td>
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<tr>
<td>gi 148380911</td>
<td>66,848</td>
<td>69</td>
<td>3</td>
<td>2</td>
<td>Chaperone protein <em>(Clostridium botulinum A)</em></td>
</tr>
</tbody>
</table>
When studying the effect that some treatments applied to \textit{C. tyrobutyricum} spores prior to inoculation have on the immune response, we could observe that heated or sonicated spores produced a higher immune response in rabbits. This difference could be explained by the modification that the exosporium proteins and spore coat proteins may undergo by those treatments, which produces an increase in the hydrophobic characteristics of spores (Wiencek et al., 1990). Borthwick, Love, McDonnell and Coakley (2005) also reported that ultrasound treatments caused a release of soluble antigens that increased the immunodetection of \textit{Bacillus subtilis} spores. The changes in the spore surface would make some epitopes more accessible, resulting in a more intense immune response in the first inoculations. However, although the titre of the serum from the rabbit inoculated with non-treated spores increased slowly with immunisations, in the fifth bleeding the titre was similar to titres of sera from rabbits immunised with non-treated spores.

The main reason for obtaining antibodies against \textit{C. tyrobutyricum} spores in our research is their future application in the development of an IMS method to concentrate and detect this microorganism. Following this objective, we also studied the possibility of applying different treatments on spores as a way of improving their immunodetection. As the results have shown, differences between non-treated or sonicated spores and heated spores were observed when using sera from the first bleeding. This result agrees with the hypothesis of a better interaction of antibodies with spore antigens that have been treated by heat due to the induction of protein disruption. However, although heating increased the immunodetection of spores when there was a low antibody titre, no differences were observed between non-treated and sonicated spores. It is possible that the intensity of sonication used in our work was not as high as previous studies (Borthwick et al., 2005), therefore proteins were not disrupted enough to improve immunodetection.

The second objective of this work was to characterise the immunodominant protein of \textit{C. tyrobutyricum} spore. Some authors have characterised the proteins of \textit{C. tyrobutyricum} vegetative cells (Bergère, Hayes, & Commissaire, 1986; Scolari et al. 1993), however, to date, no studies about spores have been reported. In order to obtain cell wall extracts, Bergère et al. (1986) used a heat treatment of 100°C during 5 min in the presence of SDS and β-mercaptoethanol. They found a major protein in the cell wall with a molecular weight of 116 kD that had not been observed in our electrophoresis pattern. However, Hayes, Commissaire and Bergère (1984) reported that, depending on the strain of \textit{C. tyrobutyricum}, the major protein of the vegetative cell extracts could be a protein with a molecular mass of 110, 116 or 125 kD, so the band that appears in our electrophoresis with an apparent molecular weight of 126 kD (Figure 4) could correspond to the 125 kD band described in the work by Hayes et al. (1984) for strain \textit{C. tyrobutyricum} 608. It has been reported that the electrophoretic pattern of the proteins from the cell wall of bacteria may even change during the sporulation process, though it has been demonstrated in studies on \textit{Bacillus} spores (Baillie & Norris, 1964; Waites, 1968) that some thermostable antigens present in the vegetative cells can also be found in the spores.

When analysing the spore extract by transfer-blotting to identify the immunogenic protein using antisera from the fifth bleeding, we found that our antisera reacted mainly against a 73-kD band. Scolari et al. (1993) analysed the proteins extracted in 8 M urea from \textit{C. tyrobutyricum} and reported that their antisera recognised five bands with molecular weights ranging from 25 to 45 kD. However,
Scolari et al. (1993) only analysed antigens from vegetative cells and this could explain the difference with our results. As it is the presence of low numbers of spores and not of vegetative cells which causes late blowing in cheese, we considered it more appropriate to study the most immunogenic protein of \textit{C. tyrobutyricum} spores. It is also remarkable that the most immunogenic protein we detected does not correspond to any of the major proteins present in the spore extract. Sharp and Poxton (1988), working with \textit{C. difficile}, also found a weakly stained band that was revealed as highly immunogenic, although in this case the major protein bands were also revealed as the major antigens.

The differences found on the recognition of the immunogenic protein by antisera from the first and the fifth bleedings would indicate that this protein is probably inside the coat structure, thus the immune response was moderate against this antigen in the first inoculations. The release of the protein caused by some treatments or simply a small change in its conformation could make this protein more effective in stimulating the immune system. This would explain the highest immunogenicity of the heated spores in the first bleeding compared to spores subjected to other treatments. In subsequent immunisations, when the immune system is already sensitised, the reactivity against the 73 kD protein increased, and the differences in immunodetection between non-treated and heat-treated spores decreased.

The analysis of proteome by mass spectrometric methods has correlated to the peptide fragments from proteins within a pre-existing database. Due to the high homology between living organisms at the molecular level, it is possible to use the available protein sequences accumulated in databases as a reference for the identification of unsequenced proteins by sequence-similarity database searching. The score obtained for the identified proteins listed in this work is higher than 60, which indicates extensive homology with the immunodominant protein. However, none of the proteins has been identified in \textit{C. tyrobutyricum} species, and in all cases, the sequence coverage is $<$10\% and the number of matching peptides $<$5. These facts would indicate that the identification of the immunodominant protein of \textit{C. tyrobutyricum} as a chaperonin is inconclusive maybe because the existing databases do not contain enough information about proteins of \textit{Clostridium} species, in particular those of \textit{C. tyrobutyricum}. Further studies would be necessary to get a better characterisation of the immunodominant protein of \textit{C. tyrobutyricum} spores, which could be useful in the development of immunological techniques to detect it.

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