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Inhibition of *Listeria monocytogenes* in Cooked Ham by Virulent Bacteriophages and Protective Cultures[▽]

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Protective cultures can be used successfully as an additional hurdle together with phages to reduce growth of *Listeria monocytogenes* on sliced cooked ham. Addition of phages resulted in a rapid 10-fold reduction of *L. monocytogenes*. After 14 to 28 days of storage, a 100-fold reduction was observed in samples with phages and protective culture compared to results for samples with phages alone.

Listeriosis in Europe has an average incidence between 2 and 10 reported cases per million population per year (7). *Listeria monocytogenes* is found in raw and ready-to-eat (RTE) products, poultry, seafood, and dairy products. A review of the incidence and transmission of *L. monocytogenes* in RTE products has been published by Lianou and Sofos (11). The USDA has implemented a "zero-tolerance" policy for *L. monocytogenes* in RTE products (2). In the European Union, the limit for common RTE foods is 100 CFU/g (1). Recently, Codex Alimentarius adopted new standards for *L. monocytogenes* in RTE foods, with a limit of 100 CFU/g in foods where *L. monocytogenes* cannot grow and absence in foods where the bacterium can grow. However, an alternative approach is accepted. Competent authorities may choose to establish and implement other validated limits (http://www.codexalimentarius.net/web/archives.jsp?lang=en, Alinorm 09/32/REP and Alinorm 09/32/13). *L. monocytogenes* in cooked products is connected with cross-contamination after heat treatment (11, 12). Bacteriophages have been successfully applied to a number of food products to reduce the level of contaminating *L. monocytogenes* (6, 8–10, 13, 14). The effect of phages varies with the type of product and is strongly dose dependent (6, 8). Active phages can be recovered from foods after long storage, but the phage particles appear to become immobilized soon after addition to nonliquid foods and therefore, due to limited diffusion, cannot infect bacteria (8). Bacteria surviving phage treatment can later grow in the product. Additional hurdles should therefore be present to inhibit later outgrowth of *L. monocytogenes*.

We have previously employed *Lactobacillus sakei* TH1 as a protective culture against *L. monocytogenes* (4, 5). Here we examine the combined use of phages and protective culture to reduce outgrowth of *L. monocytogenes* on cooked ham.

Rifampin (rifampicin)-resistant mutants of *L. monocytogenes* 2230/92 serotype 1, implicated in a listeriosis outbreak in Norway (12), and *L. monocytogenes* 167 serotype 4b were grown overnight in brain heart infusion (Difco Laboratories, Detroit, MI) at 37°C without shaking and stored at 4°C for 24 h (3–5). Cells were diluted in 0.9% NaCl and plated on brain heart infusion agar with 200 μg/ml rifampin. *L. sakei* TH1 was grown at 30°C in MRS (de Man, Rogosa, Sharpe) broth (CM 359; Oxoid, Hampshire, England) (pH 6.2) and plated on MRS agar (5). Listex P100 phages, 2×10^{11} PFU/ml, were from EBI Food Safety (Wageningen, The Netherlands).

Ten-gram slices of hams with 2.3% NaCl and 0.01% disodium diphosphate (pH 6.2; $a_w > 0.97$), made at Nofima's pilot plant (Aas, Norway), were inoculated with a cold-adapted 1:1 mixture of *L. monocytogenes* 2230/92 and 167. Bacteria were spread in 100 μl 0.9% NaCl over the 80-cm^2 surface area of each slice to 10^3 CFU/cm^2 using a bent glass rod. After 1 h at 20°C, phages (5×10^7 PFU/cm^2 in a total volume of 100 μl) were spread over the same surface. After one additional hour, 10^3 CFU/cm^2 *L. sakei* TH1 in 100 μl 0.9% NaCl was added where appropriate. The slices were vacuum packed and stored at 10°C. Growth was measured before and after spiking and at 0, 3, 7, 14, and 28 days after homogenizing the slices in 100 ml 0.9% NaCl in a Stomacher homogenizer. No lactic acid bacteria were detected in uninoculated samples. Experiments were performed with three parallel samples. *L. monocytogenes* alone grew from 10^4 CFU/g at the onset of the experiment to 10^7 CFU/g the first 7 days, reached 2×10^8 CFU/g after 14 days, and remained unchanged thereafter (Fig. 1). In samples with both *L. monocytogenes* and phages, a rapid 1-log reduction in *L. monocytogenes* was observed. Surviving *L. monocytogenes*, however, grew as well as that in the phage-free controls, reaching $>10^7$ CFU/g after 14 days. In samples where both P100 phages and *L. sakei* TH1 were added, the same initial reduction of *L. monocytogenes* was observed, but the later outgrowth was reduced by the fast-growing lactic acid bacteria and the *L. monocytogenes* levels were 2 logs lower than those with P100 phages alone after 28 days of incubation. The phages did not influence the growth and survival of *L. sakei* TH1. During the 28 days of storage, the pH changed from 6.20 to 6.05 in samples with *L. monocytogenes* and to 6.00 in samples with both *L. monocytogenes* and *L. sakei* TH1. The results were reproduced in a separate repetition of the experiment at 10°C (not shown).

The effect of the protective culture was dose dependent when 10^4 CFU/g and 10^6 CFU/g of *L. sakei* TH1 were added to slices of ham (Fig. 2). *L. monocytogenes* alone grew to 2×10^8 CFU/g after 14 days. When *L. sakei* TH1 was added at a low concentration (10^4 CFU/g), *L. monocytogenes* grew to approx-

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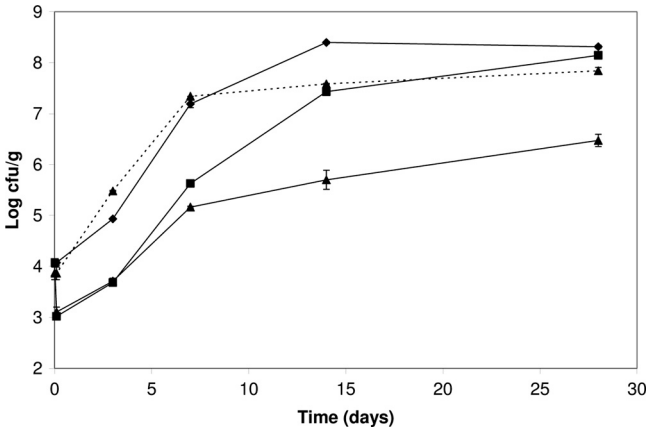

FIG. 1. Inhibition of *L. monocytogenes* in cooked ham with bacteriophages and protective culture at 10°C. Sliced ham was inoculated with 10^3 CFU/cm^2 (corresponding to approximately 10^4 CFU/g) *L. monocytogenes* (◆), *L. monocytogenes* and 5×10^7 PFU/cm^2 P100 phages (■), or *L. monocytogenes*, 5×10^7 PFU/cm^2 P100 phages, and 10^3 CFU/cm^2 (approximately 10^4 CFU/g) protective-culture *L. sakei* TH1 (▲) and stored at 10°C. Growth of *L. sakei* TH1 is shown by the broken line.

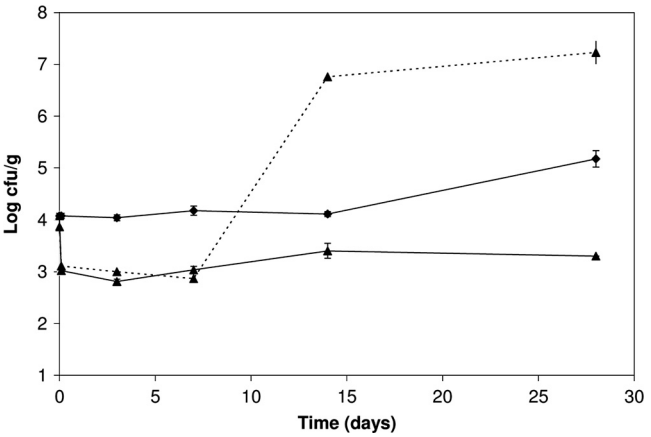

FIG. 3. Inhibition of *L. monocytogenes* in cooked ham with bacteriophages and protective culture at 4°C. Sliced ham was inoculated with 10^3 CFU/cm^2 (corresponding to approximately 10^4 CFU/g) *L. monocytogenes* (◆) or *L. monocytogenes*, 5×10^7 PFU/cm^2 P100 phages, and 10^4 CFU/g (10^3 CFU/cm^2) protective-culture, *L. sakei* TH1 (▲) and stored at 4°C. Growth of *L. sakei* TH1 is shown by the broken line.

imately 4×10^6 CFU/g, while when *L. sakei* TH1 was added at a high concentration, *L. monocytogenes* levels were 1 to 2 logs lower. The pHs in the low- and high-inoculum hams were reduced from the initial 6.20 to 6.16 and 6.02, respectively, at day 28. For hams stored at 4°C, slow growth of *L. monocytogenes* occurred between days 14 and 28 from 10^4 to 10^5 CFU/g ($P = 0.003$) (Fig. 3). With phages and *L. sakei* TH1 added, a rapid 1-log reduction of *L. monocytogenes* was observed due to the phage attack, and no growth was observed during the 28-day storage period. The *L. sakei* TH1 strain showed a longer lag phase at this low temperature but nevertheless reached 10^7 CFU/g at day 14 and thereby inhibited any growth of *L. monocytogenes*.

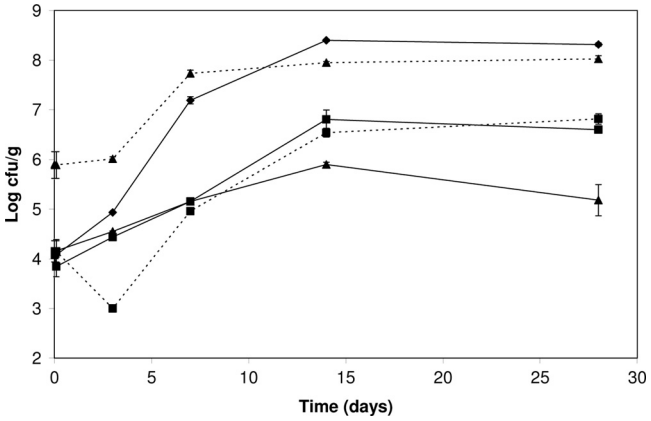

FIG. 2. Inhibition of *L. monocytogenes* in cooked ham inoculated with large or small amounts of protective culture at 10°C. Sliced ham was inoculated with 10^3 CFU/cm^2 (corresponding to approximately 10^4 CFU/g) *L. monocytogenes* (◆), *L. monocytogenes* and 10^6 CFU/g (10^5 CFU/cm^2) *L. sakei* TH1 (▲), or *L. monocytogenes* and 10^4 CFU/g (10^3 CFU/cm^2) *L. sakei* TH1 (■) and stored at 10°C. Growth of *L. sakei* TH1 is shown by broken lines. The *L. monocytogenes* control is the same control as in Fig. 1.

Since *L. sakei* TH1 grows well at low temperatures, prevents growth of *L. monocytogenes*, and has no negative influence on the organoleptic properties of ham (4, 5), it can successfully be employed as an additional hurdle together with phages.

We here chose to perform the storage experiments under "worst-case" conditions. Generally, the contamination levels of *L. monocytogenes* are lower than in our setup, in the range of 10 to 100 CFU/g (see reference 11 and references therein). Since *L. sakei* TH1 grows well at low temperatures (Fig. 3), its selective advantage will be greater at 4°C than at abuse temperatures. From the above, it is evident that it is possible to optimize *L. monocytogenes* inhibition by increasing both the phage titer and the starting amount of protective culture. An enhanced effect may also be experienced by modifying phage application, e.g., by using larger liquid volumes (6, 8).

Emergence of resistant *L. monocytogenes* may be a potential problem when treating foods with phages. No emergence of resistance has been detected after phage treatment (6, 8). Such strategies as use of phage mixtures, phage rotation schemes, and treatment of products immediately prior to packaging may reduce eventual resistance problems (8). Some *L. monocytogenes* strains are naturally phage resistant (6). In these cases, a protective culture still constitutes a powerful hurdle.

In conclusion, we have shown here that by applying phages and protective culture as two independent hurdles, it is possible to both reduce the number of *L. monocytogenes* bacteria on a product and inhibit outgrowth of eventual remaining surviving cells. This is a general method that can potentially be applied to different foods where there is a potential risk for growth of *L. monocytogenes*, provided a suitable protective culture is available.

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