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Application of phages prev outgrowth of *Listeria* on ch

A successful strategy to ensure food safety, requires food producing companies to treat food safety as an integral responsibility of the manufacturing and supply chain. In this approach, a policy effectively aligning the GMP, GHP, QA/QC and HACCP programmes, is implemented to reduce the risk of contamination by *Listeria monocytogenes.*

The moment a *Listeria monocytogenes*-contaminated cheese is discovered (pathogen alert), a very strict (alert triggered) protocol is observed, requiring substantial efforts and resources from management and production plant employees, to prevent this contamination from spreading inside the premises, and to guarantee that contaminated cheeses do not reach the retail environment, or worse, the consumer.

The preventive application of anti-Listeria phages, can significantly enhance the efficacy of this strategy by

1. reducing the number of alerts and
2. effectively preventing the cheese itself from becoming a source of re-contamination for people, equipment and the production environment at large. By the latter effect, a threatening gap in existing HACCP programmes can be closed.

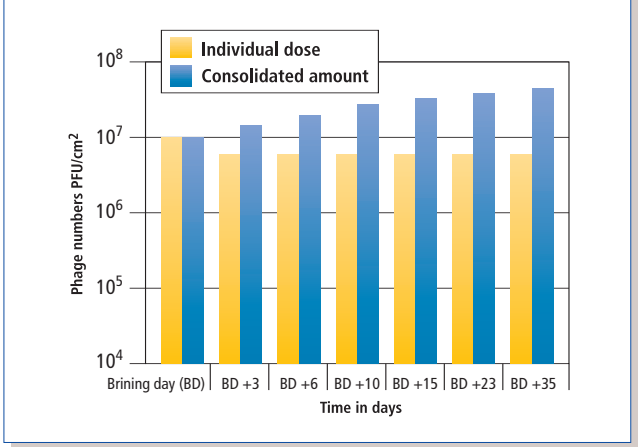
Bacteriophages in use to eradicate undesirable and pathogenic micro-organisms in food production increasingly present themselves as a useful extension of the instruments food companies deploy to control food safety issues and are expected to become an industry standard.

For cheese manufacturers, control of Listeria is one of the major challenges. The professional industrial cheese producers have adopted an approach to tackle this food safety threat as an integrated manufacturing supply chain responsibility. The most important contributions to the actual food safety situation come from the application and rigorous maintenance of professional GMP, GHP and HACCP programmes.

These measures effectively focus on avoiding the introduction of new *Listeria monocytognes*-contaminations from equipment, people and the production environment to the ripening cheeses. In case a *Listeria monocytogenes* (Lm) strain does end up on a cheese, a series of actions is required to avoid contamination.

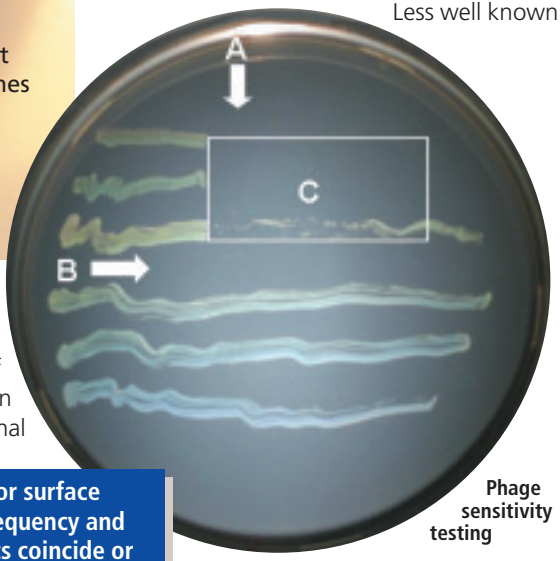
However, in the meantime the contaminated cheeses may have become a relevant source of re-contamination of equipment, people and the environment, hereby devaluating earlier investments in food safety. The application of the Listex P100 phage culture can prevent the outgrowth of occasional Listeria on cheese. Elimination of cheese as a source of re-contamination not only results in a safer finished product, but also increases the return on previous food safety investments.

Figure 1: Listex P100 application scheme for surface ripened cheese; typical data on dosage, frequency and timing of the treatments. Phage treatments coincide or are combined with the regular smearing treatments



Phages in cheese

Phages have always been present in cheese. A well known source is the contamination of the starter culture, resulting in slower acidification during fermentation of milk during curd making. Less well known is the contribution to an acceleration of the cheese ripening, which may result from phage induced lysis of ripening bacteria. This phenomenon, although difficult to quantify, is thought to be more frequently associated with the use of traditional starter cultures and whey starters in the production process of more artisanal cheese varieties.



Phage sensitivity testing

A = phage lane
B = listeria lane
C = sensitivity area

Thirdly phages are more prevalent in raw milk cheese than in industrial cheeses due to the fact

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that cheese milk already carries a natural phage load.

Yet another source of phages is found in the normal factory environment in which the cheese is either produced or stored during ripening. This is particularly relevant for surface ripened and smeared cheese types. In these cheeses, phages which target bacteria from the smear-cultures can be present, as well as phages derived from the bacteria in the natural contaminating flora of the ripening room.

Listex P100: broad spectrum of anti-Listeria phage culture

The P100 phage is a rare, polyvalent anti-Listeria phage, which has been selected from a natural source. The phage is characterised by its broad spectrum against the food borne pathogen *Listeria monocytogenes*. When tested for its suitability for food applications, phage P100 demonstrated to be effective against > 95 % of tested strains of Lm. The large majority of tested Lm-strains were taken from food sources. The P100 phage is a

EBI Food Safety

EBI Food Safety is a privately held Dutch Life Sciences company, focused on the development and commercialisation of new anti-bacterial agents, based on its bacteriophage technology. The company's scientific network includes collaborations with universities and research centres in The Netherlands, Switzerland, Spain, Belgium, Portugal and Italy.
The company is viewed as product leader in the field of bacteriophage technology for food applications. In September 2006, EBI Food Safety was awarded the Frost & Sullivan Technology Innovation Award 2006 for its contributions to the food safety arena.

For more information, also visit www.ebifoodsafety.com

Presence and function of phages in nature

Phages serve as the natural counterbalance to bacteria and herewith have become the most ubiquitous source of life on earth. Without phages in our environment, bacteria would soon overwhelm our planet. It is likely that every bacterial strain has at least one phage-counterpart for which the bacterial strain serves as a natural host. Phages are by definition highly "species specific", and every phage has at least one bacterial strain which serves as its source for replication. The phage relies on this host strain for its survival in nature.

purely lytic phage and has been well characterised; its DNA-sequence has been published and specifies no undesirable genes and the phage is therefore known to be safe (Regulatory Journal of Toxicology and Pharmacology, Sept. 2005). The phage DNA cannot integrate into the host-DNA. Since part of the spectrum also covers a number of members of the family of Class I (safe) micro-organism *Listeria innocua* (Li), a production route for a safe fermentation product could be successfully developed. Following

a natural fermentation with Li, upon infection of the host bacterium, P100 is replicated and set free in the fermentation medium. The subsequent separation and purification of the fermentation medium, yields Listex P100 as a safe culture of the broad-spectrum P100 phage for use in food.

Application of P100 in the production of surface smeared cheese

The working mechanism of phages enables a preventative application in cheese making, aimed specifically at stopping and killing Listeria infections at the earliest possible stage. If a Listeria strain reaches a cheese despite all properly implemented and maintained GHP, GMP and HACCP measures, it is essential to prevent the initial infection from growing to high numbers.

Only by doing so, can it be avoided that the contaminated cheese becomes a significant source of re-contamination of the production environment, an event which could lead to an explosion of Lm. Within well performing HACCP-programmes, this is one of the most feared issues, which can destroy the benefits of weeks or months of investments in safety and hygiene.

Listex P100 is not to be applied curatively. Not only may it fail to show efficacy but, more importantly, the application of phages is to be regarded as complimentary to proper GMP and GHP programmes, and not as an alternative to it. The evidence collected by EBI Food Safety in several pilot and industrial scale trials, indicates that integration with the smear-treatment, where the first phage-treatment is applied shortly after brining, is effective in the control of an artificially applied contamination with *Listeria monocytogenes*, and prevents the treated cheeses from becoming a source of recontamination.

Spectrum, sensitivity and polyvalence

The spectrum of a phage is defined by the host range of bacteria which are susceptible to infection by that specific phage. A phage with a broad spectrum has a high number of different bacterial strains from one specific group or family (i. e. *Listeria innocua* or *Listeria monocytogenes*) which it can use for its replication.
The sensitivity of a bacterial host strain is determined by the fact that the host strain can be infected by one or more phage(s). This capability of a bacterial strain is a property of that particular bacterial strain. A polyvalent phage has a broad spectrum, covering more than one bacterial host strain; the spectrum of P100 is very broad; >95 % of tested *Listeria monocytogenes*-strains, of food origin, are sensitive (diagram: EBI's ABC to sensitivity: a host range test to determine whether a test strain falls inside the spectrum of P100).

How to apply Listex P 100 effectively

The main features of a successful integration in cheese making processes deal with the application of the right amount of P100 phages at the correct moment and with the desired frequency.

A typical application scheme for Listex P100 phages, with multiple applications, is shown in the bar diagram. Starting with an initial treatment with approximately 1 x 10^7 PFU/cm^2 at the exit of the brine, subsequent follow up treatments are periodically given. The dosage at the follow up treatment is chosen to inoculate again at a level of approximately 5 to 9 x10^6 PFU/cm^2.

The initial treatment is performed either by spraying the phage culture onto the cheese surface or by dipping the cheese in a phage suspension. Another option is to blend the phage culture together with the smear culture. Follow up treatments are given for instance by treating the cheese with a combined smear-phage-solution or by spraying the designated amount of phage onto the cheese at the exit of the machine.

For an optimal result, in typical applications, overall surface concentrations of phages range from approximately 2 x 10^7 PFU/cm^2 in short ripening cheeses (10 to 20 days) to approximately 7 x 10^7 PFU/cm^2 for longer ripening cheeses (two weeks to two months).

The frequency of treatment varies from two to three treatments in Limburger and Munster cheese to about a frequency of five to ten treatments in the manufacturing process of longer-ripening cheeses, such as Bergkaese, Tilsiter, Appenzeller or Raclette.

In the production of white and blue mould cheeses, P100 phages can be applied without adverse effect until the surface mould has established itself on the cheese surface. As a general principle, one can reduce the dosage rate of the follow up treatments somewhat with an increased frequency of the treatment.

The overall object is to maintain a surface concentration of phages at a functional and acceptable level; i. e. in excess of 1,5 x 10^7 PFU/cm^2.

The P100 phages do not interfere with ripening and have no influence on taste, flavour, appearance or consistency of the cheese. The phages do not affect the surface smear bacteria or yeasts, and the P100-phages are temperature- and salt-tolerant within the normal ranges seen in cheese making.

Phages in food production

Listex P100 is a culture of safe micro-organisms. Within the European Union, the use of safe micro-organisms as processing aids is free and unrestricted. In the USA, phages have already been approved under 21 CFR § 172.785 for surface treatment of meat and poultry products. Based on EBI Food Safety's successful completion of the relevant safety studies, a unanimous positive expert panel opinion for GRAS and FDA notification was given.

EBI Food Safety participates in several EU-projects regarding the appropriate use in foods of bacteriophages with the objective to eradicate other pathogens, such as Campylobacter, E.coli O 157, Salmonella, Shigella etc. from the food chain. Listex P100 is one of the first concrete examples resulting from this new activity of the fermentation industry.