

White Paper

Thermal Control of Dairy Bacteriophage



Table of Contents

Executive Summary	3
Introduction to SPX Flow Technology	3
Vision and commitment	3
Customer focus	3
Introduction to bacteriophage	4
Morphology and taxonomy	4
Life cycles	6
Bacteriophage in the dairy	8
Controlling bacteriophage in the dairy environment	8
General recommendations	8
Killing phage by heat treatment	8
Logarithmic reduction	8
Phage lethal time and temperature combinations achieved with SPX Flow Technologies	9
Thermal treatment of whey protein concentrates and control of bacteriophage using APV LeanCreme™ technology	9
References	12

Executive Summary

The APV LeanCreme™ technology involves the addition of microparticulated whey protein concentrate to be added to cheese milk as a means of increasing cheese yield and/or to reduce the fat content of the cheese without compromising product taste, flavour and texture.

This white paper describes bacteriophage and explains how to reduce the possibility of phage attack on the cheese starter culture whilst maintaining the essential functionality of APV LeanCreme™. It is demonstrated that the time/temperature conditions applied during the microparticulation process are sufficient to destroy most of the less heat resistant bacteriophage encountered in dairies. However, some high heat resistant strains being able to survive temperatures of 115°C. If there is a concern for these specific strains then LeanCreme has to be combined with non-heated based methods to prevent phage contamination.

As a matter of fact, it is not recommended to apply temperatures above 85°C to 90°C in order to destroy all phage as it will cause excessive denaturation of the microparticulated whey protein with the result that less protein will be incorporated into the cheese.

Whatever heat treatment is applied it is a general recommendation to combine the heat treatment with e.g. culture rotation and chemical cleaning to limit the presence of phage. It is not just a matter of heat treatment. By having a low growth of phage as a starting point for a stream means that a gentler heat treatment is required (lower logarithmic reduction). The quality of the final product in terms of microbiology, taste and texture can hereby be preserved.

Introduction to SPX Flow Technology

Vision and commitment

SPX's Flow Technology segment designs, manufactures and markets process engineering and automation solutions to the dairy, food, beverage, marine, pharmaceutical and personal care industries through its global operations.

We are committed to helping our customers all over the world to improve the performance and profitability of their manufacturing plant and processes. We achieve this by offering a wide range of products and solutions from engineered components to design of complete process plants supported by world leading applications and development expertise.

We continue to help our customers optimise the performance and profitability of their plant throughout its service life with support services tailored to their individual needs through a coordinated customer service and spare parts network.

Customer focus

Founded in 1910, APV, an SPX Brand, has pioneered groundbreaking technologies over more than a century, setting the standards of the modern processing industry.

Continuous research and development based on customer needs and an ability to visualise future process requirements drives continued mutual growth.



Introduction to bacteriophage

Fermentation defects or failures in products like cheese and fermented milk products can result in changes in texture, gas formation and flavour of the products. Fermentation failures can, among others issues, be caused by bacteriophage contamination and therefore understanding the role of bacteriophage and what can be done to avoid them, is important. One way of controlling bacteriophage is by thermal treatment.

Bacteriophage are a natural part of our surroundings and are amongst the most common organisms on Earth, e.g. 10 million bacteriophage exist in one single millilitre of sea water and exist in general in the biosphere and therefore also in the air (<http://en.Wikipedia.org/wiki/Bacteriophage>). This partly explains why bacteriophage cannot be fully avoided. In the case of dairies some species of bacteriophage are not wanted due to risk of fermentation failures. However, a lot can be done to limit the occurrence.

Below is an electron micrograph showing a number of bacteriophage invading a bacterium.



Fig. 1: Electron micrograph showing bacteriophage invading a bacterium, (<http://en.wikipedia.org/wiki/Bacteriophage>)

Morphology and taxonomy

Βακτηριου (bakterion) = bacteria
Φαγοζ (phágos) = to eat

Bacteriophage is a virus that replicates itself using hosting bacteria. The term is commonly used in its shortened form, phage. Phage comes from the Greek work "phagein" and means "to eat" - bacteriophage can literally be translated to "to eat bacteria" (<http://en.wikipedia.org/wiki/Bacteriophage>).



Fig. 2a: An electron micrograph (http://www.mansfield.ohio-state.edu/~sabedon/beg_phage_images.htm and <http://en.wikipedia.org/wiki/Bacteriophage>)

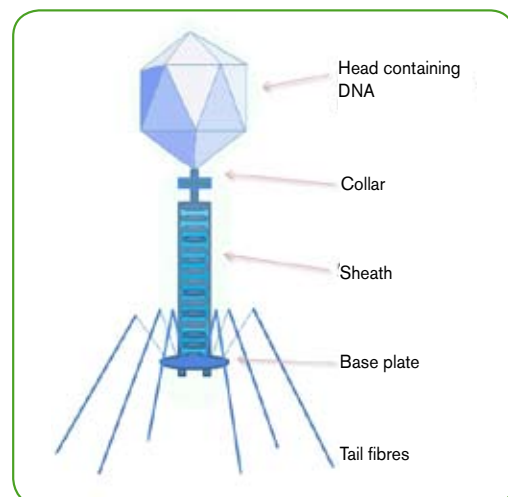


Fig. 2b: A schematic illustration of bacteriophage, (http://www.mansfield.ohio-state.edu/~sabedon/beg_phage_images.htm and <http://en.wikipedia.org/wiki/Bacteriophage>)

Phage infect bacteria and the properties of the bacteria can be changed or even destroyed. These changes are often not harmful and may even be beneficial. However, in fermented dairy products like cheese and yoghurt where starter cultures are used, such changes are not wanted since the fermentation process can be retarded or completely stopped.

Bacteriophage come in many different sizes and shapes. The basic structure is illustrated in the electron micrograph and schematic drawing on figs. 2a and 2b.

All phage have a head which contains the DNA or RNA genetic material. Many but not all phage have tails. The base plate and tail fibres are involved in binding of the phage to the bacterial cell. Bacteriophage are often much smaller than bacteria – usually between 20 to 200 nm (Madigan et al., 2000).

For practical reasons, bacteriophage are classified according to the International Committee on Taxonomy of Viruses (ICTV). As can be seen in Table 1 below, the morphology determines the classification, e.g. if a phage has tail or not.


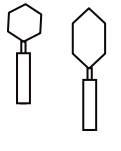

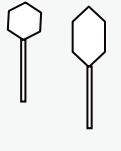

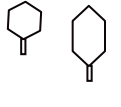












Bradley (1967)		Ackermann & DuBow (1987)		ICTV (1982)	
Name	Morphology	Name	Morphology	Family	Genus
A		A1, A2, A3		<i>Myoviridae</i>	-
B		B1, B2, B3		<i>Siphoviridae</i>	-
C		C1, C2, C3		<i>Podoviridae</i>	-
D		D1		<i>Microviridae</i>	<i>Microvirus</i>
		D2		<i>not classified</i>	-
		D3		<i>Corticoviridae</i>	<i>Corticovirus</i>
		D4		<i>Tectiviridae</i>	<i>Tectivirus</i>
E		E1		<i>Leviviridae</i>	<i>Levivirus</i>
		E2		<i>Cystoviridae</i>	<i>Cystovirus</i>
F		F1		<i>Inoviridae</i>	<i>Inovirus</i>
		F2			<i>Plectovirus</i>
-		G		<i>Plasmaviridae</i>	<i>Plasmavirus</i>

Table 1: Comparison of phage classification by Bradley (1967), by Ackermann and DuBow (1987) and by the International Committee on Taxonomy of Viruses (ICTV, 1982)

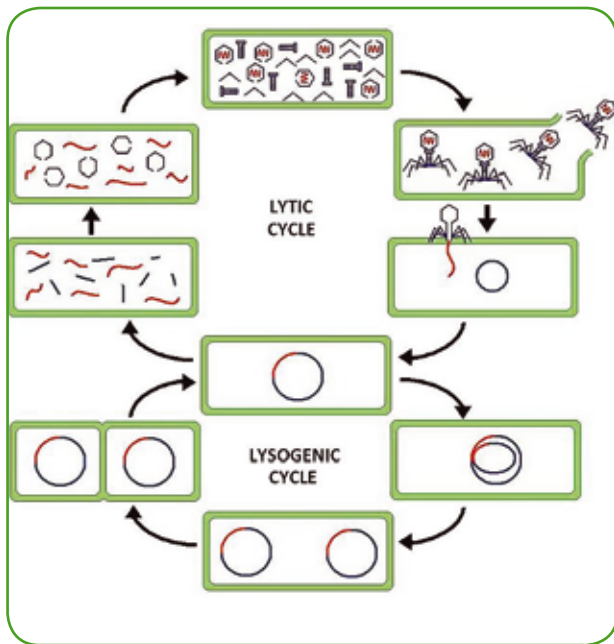


Fig. 3. Lytic and lysogenic Bacteriophage infection.
http://en.wikipedia.org/wiki/Lytic_cycle

Life cycles

The process of “infection” is where a virus genome (e.g. DNA) is introduced into a host cell and reproduces (Madigan et al., 2000).

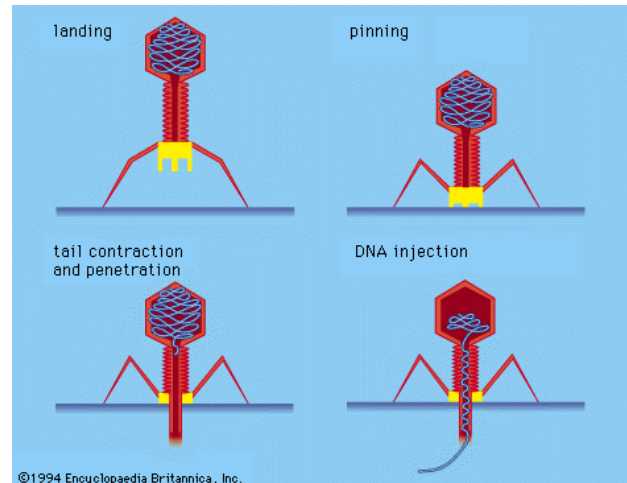
Phage can reproduce via two different life cycles - the lytic and the lysogenic cycle. See fig. 3.

Some phage replicate via the lytic cycle and are normally referred to as “virulent”. In the lytic life cycle, the phage open (lyse) the bacterial cell resulting in destruction.

When virulent bacteriophage infect bacteria, sub-units of phage are synthesised, assemble and finally mature phage is released. The host cell is killed. The released phage are now ready to infect a new bacterium.

Fig. 4 illustrates the process of the DNA injection in detail. First step is the landing of the phage onto the living cell. The landing can only occur if specific bacteria cell surface components, called receptors, match the tail fibres of the phage. There is a high specificity in this landing step like the match of a key and lock.

One way for the bacteria to defend it self is to have no receptors. If the receptors exist, the second step is the phage pinning (attaching) before it penetrates the



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Fig 4.: Schematic showing the process of bacteriophage hosting bacteria,
http://www.mansfield.ohio-state.edu/~sabedon/beg_phage_images.htm

tail into the host cell. Finally the DNA of the phage is injected into the cell.

Some bacteriophage can propagate by both the lytic and the lysogenic cycle, and are normally referred to as “temperate” phage. In the lysogenic cycle, the host and phage form a symbiosis. The phage genome is integrated into the host genome and replicated with it. At some point in time, phage is released and enters into the lytic phase. The majority of *Lactococcus lactis* phage strains are lysogenic.

The period from landing of phage to assembly and release of phage is called the latent period. The burst size is the number of phage released from each bacterium. This number can vary from 200 to less than 10 and is phage specific.

The consequence of infected bacteria in a fermentation process can be slow acidification or stagnancy. In practice, the fermentation period has to be extended to reach the normal fermentation pH. The fermentation curves differ greatly if the burst size is high. On the other hand, if the burst size is low the fermentation curve may not differ significantly at first sight. Schematic illustration of the influence of burst size on the fermentation curve can be seen in the graphs.

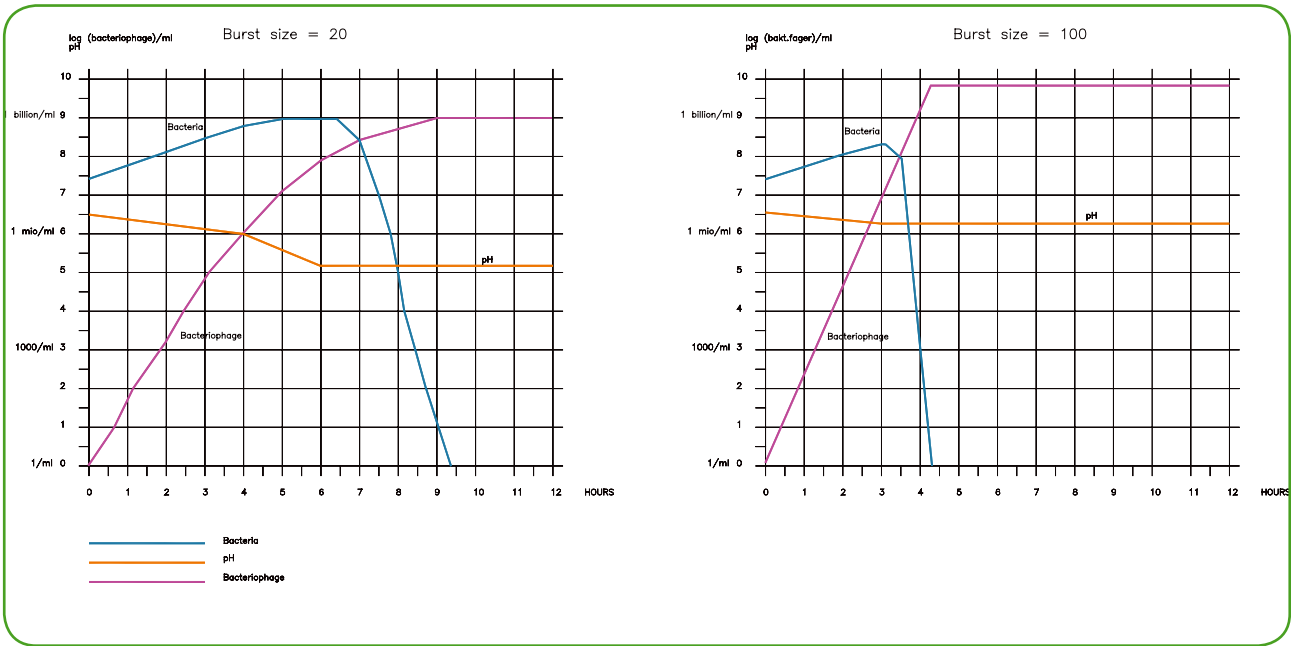


Fig. 5. Examples of computer simulated fermentation curves with bacteria infected by phage with burst sizes of 20 and 100 (IDF No. 308, 1995)

As can be seen the number of bacteria decreases as the number of bacteriophage increases. In these examples a burst size of 20 results in prolonged fermentation time and burst size of 100 results in stagnation of pH.

In the graphs in fig. 6, the burst size is just 7, which is low. At day 1, the fermentation seems normal.

However, at day two the starting number of phage is higher with the result that the fermentation curve stagnates.

Bacteriophage tend to mutate spontaneously in response to changes in environment which can make them aggressive e.g. if starter culture rotation is not done.

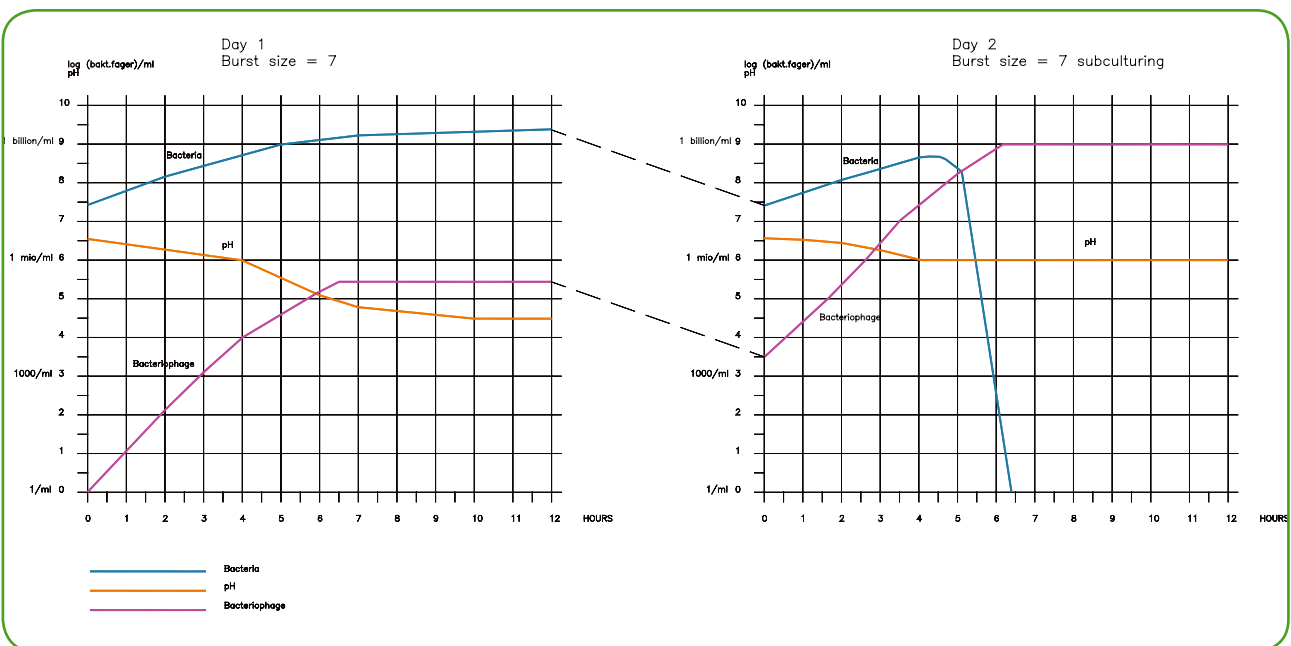


Fig. 6. Computer simulated effect of time on fermentation curves with bacteria infected by phage with a burst size of 7 (IDF No. 308, 1995)

Bacteriophage in the dairy

It has been estimated that dairies experience phage-induced fermentation failure in 0.1. – 10% of the batches. 95% of the phage problems in dairies are caused by the phage species of 936, P335 and C2.

The most important and most widespread phage to *Lactococcus lactis* is P008. It is therefore the most investigated phage for this bacterium. The latency period and burst size are dependant on the incubation conditions. It has been found that under optimal conditions, the latency period is 24 to 37 min and the burst size is 7 to 22. In other words one P008 phage usually takes 30 minutes to replicate it self to 7 to 22 phage. This is a rather quick replication and is even faster than the host bacterium itself (Müller-Merbach 2007).

Phage P008 is accepted as the official type phage for *Lactococcus lactis* phage species 936 representing the Siphoviridae family of morphotype B1. P008 is approved by the Lactococcal and Streptococcal Phage Study Group, Bacterial Virus Subcommittee, ICTV as the test phage in the European standard CEN/TC 216/WG3 N 42: "Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas.

P1532 and P680 are the most heat resistant Lactococcal bacteriophage in milk systems investigated until now. Both of them belong to the *Lactococcus lactis* phage and the 936 species (Müller-Merbach 2007). P1532 is extremely heat stable but also very rare. P1532 was detected in 0.7 % of investigated dairies and is therefore extremely rare (Dogan et al., 2008).

Phage of the host *Lactococcus lactis* and *Lactobacillus helveticus* bacteria is known to be relatively stable compared to *Lactobacillus delbrueckii* and *Streptococcus thermophilus* phage.

Controlling bacteriophage in the dairy environment

Phage enter the dairy via raw milk and whey. Some phage may survive normal pasteurisation. Inside the dairy they are mainly spread in the whey and recirculation of whey cream can potentially accelerate phage contamination problems. Reincorporation of milk components must therefore, be handled under carefully controlled conditions.

The presence of phage cannot be avoided but a lot can be done to limit their presence. Below are general recommendations as to how to avoid bacteriophage contamination.

General recommendations

- Culture rotation and use of highly concentrated direct starters (deep frozen or spray dried)
- Apply an efficient heat treatment to the whey
- Hygienic design of plant, e.g. avoid dead ends and reflux
- Handling of whey and whey plant. Personnel working with whey should preferably not work with the starter culture and should not be in contact with the fermentation process. The fermentation process should, as far as possible be a closed process
- Sufficient and regular cleaning, and disinfection with appropriate agents
- Handling of air with use of high efficiency particulate air filtration of incoming air and slight overpressure in processing rooms
- Break the reincorporation of by products into the milk regularly (e.g. whey and whey cream)

Killing phage by heat treatment

Killing and inactivation of bacteriophage infecting lactic acid bacteria has been of interest ever since phage were observed and caused fermentation failures in milk. Literature information has been controversial. However, newly extensive investigations provide data to calculate process conditions that assure adequate phage destruction to prevent fermentation failure. Whey can carry up to 10^7 to 10^9 PFU/ml (Atamer et al., 2009). Plaque Forming Units is a measure of the number of particles capable of forming plaques per unit volume. It is a functional measurement rather than a measurement of the absolute quantity of phage.

Logarithmic reduction

When micro-organisms are exposed to heat treatment, not all of them are killed at once.

However, in a given period of time a certain number are killed whilst the remainder survives. If the surviving micro-organisms are once more exposed to the temperature treatment for the same period of time an equal proportion will be killed. On this basis, the lethal effect of sterilisation can be expressed mathematically as a logarithmic function having a first order kinetic.

$$K \cdot t = \log N/N_t$$

Where

N = number of micro-organisms/bacteriophage

N_t = number of micro-organisms/bacteriophage present after a given time of treatment (t)

K = constant

t = time of treatment

A logarithmic function can never reach zero, which means that 100 % elimination is impossible to

achieve. Therefore the more workable concept of “sterilising effect” or “commercial sterility” is commonly applied. Table 2 shows an example of the influence of log reductions on survivors and killed phage. In this case the starting point is 100,000,000 (10⁸) of phage. Raw milk might have up to 10³ PFU/ml.

Having a log reduction of 8 in this example gives 1 surviving phage out of 100,000,000.

LOG REDUC-TION	SURVIVORS	KILLED PHAGE	UNIT OF TIME
0	100.000.000	0	0
1	10.000.000	90.000.000	1
2	1.000.000	99.000.000	2
3	100.000	99.900.000	3
4	10.000	99.990.000	4
5	1.000	99.999.000	5
6	100	99.999.900	6
7	10	99.999.990	7
8	1	99.999.999	8
9	0,1	99.999.999,9	9
10	0,01	99.999.999,99	10
11	0,001	99.999.999,999	11
12	0,0001	99.999.999,9999	12
13	0,00001	99.999.999,99999	13

Table 2. Overview of link between log reduction, surviving phage, killed phage and unit of time for a constant heat treatment temperature.

Phage lethal time and temperature combinations achieved with SPX Flow Technologies

In terms of phage risk, thermal treatment has the potential to increase the safety of fermentation process. To determine optimal thermal treatment for a specific case it should be more effective than a normal pasteurisation but less destructive than that required for heat sterilisation.

The time temperature graph on the next page shows the so called “lines of equal effect”. Lines of equal effect indicate combinations of time and temperature which lead to an equal effect of a reaction, e.g. an equal degree of phage inactivation or destruction of vitamin B1 (thiamine). Lines for the widespread phage P008 and for the rare and extremely heat stable phage P1532 are given for log 9 reductions. In an investigation 0.7 of samples from dairies were infected by P1532, so this is an extremely rare phage (Dogan et al., 2008). Log 9 reduction is usually sufficient for concentrated milk components like whey

protein concentrates (WPC's) as WPC often has a higher level of bacteriophage than raw milk. Referring to table of log reduction it can be seen that the initial level of bacteriophage is important in determining the heat treatment that is given.

A general rule is to aim for a threshold of 1 PFU/ml on the basis of the rate of phage reproduction. Assuming a reproduction according to phage P008 and optimum growth conditions, the phage titre will increase from 1 PFU/ml to a maximum of 10⁷ PFU/ml during a 4 hour long fermentation. This should not put the fermentation at risk yet (Müller-Merbach, 2007).

According to fig. 7, a 9-log reduction of the commonly found phage P008 can be reached with temperature and time combination ranging from 70°C for 20 min to 90°C for 1 sec. and therefore pasteurisation is normally not sufficient. A log reduction of 9 is a high reduction

Other milk components are also affected by a given temperature and time as can be seen in the temperature time graph. The challenge is to find the optimal time and temperature for a given process without destroying the end product taste and texture.

As an example, the loss of vitamin B1 and denaturation of whey proteins can be mentioned. Vitamin B1 is normally not wanted to be lost, since it is an important vitamin. In general, short time and higher temperature is most gentle to the product compared to long time and lower temperature.

Thermal treatment of whey protein concentrates and control of bacteriophage using APV LeanCreme™ technology

The APV LeanCreme™ process is a thermal treatment of WPC (whey protein concentrate) with scrape surface heat exchangers realising the denaturation and the microparticulation of the whey proteins in the very same steps. The microparticles formed with LeanCream have an exceptionally high functionality, water binding ability and when added back to the cheese milk they will stay trapped in the cheese curd (Steffl, 1999; Spiegel, 1999). However, since whey protein concentrate can potentially contain bacteriophage, it is important to control the phage by thermal treatment before adding it to the cheese milk in addition to the general recommendations previously mentioned. The block flow diagram of a typical cheese manufacturing plant Fig. 8 shows examples of the flow of whey in a typical facility.

The combination of time and temperature of the APV LeanCreme™ technology is flexible and can easily be changed. Typical optimal holding time is 60 to 120 seconds and typical optimal temperatures are in the range of 82°C to 88°C.

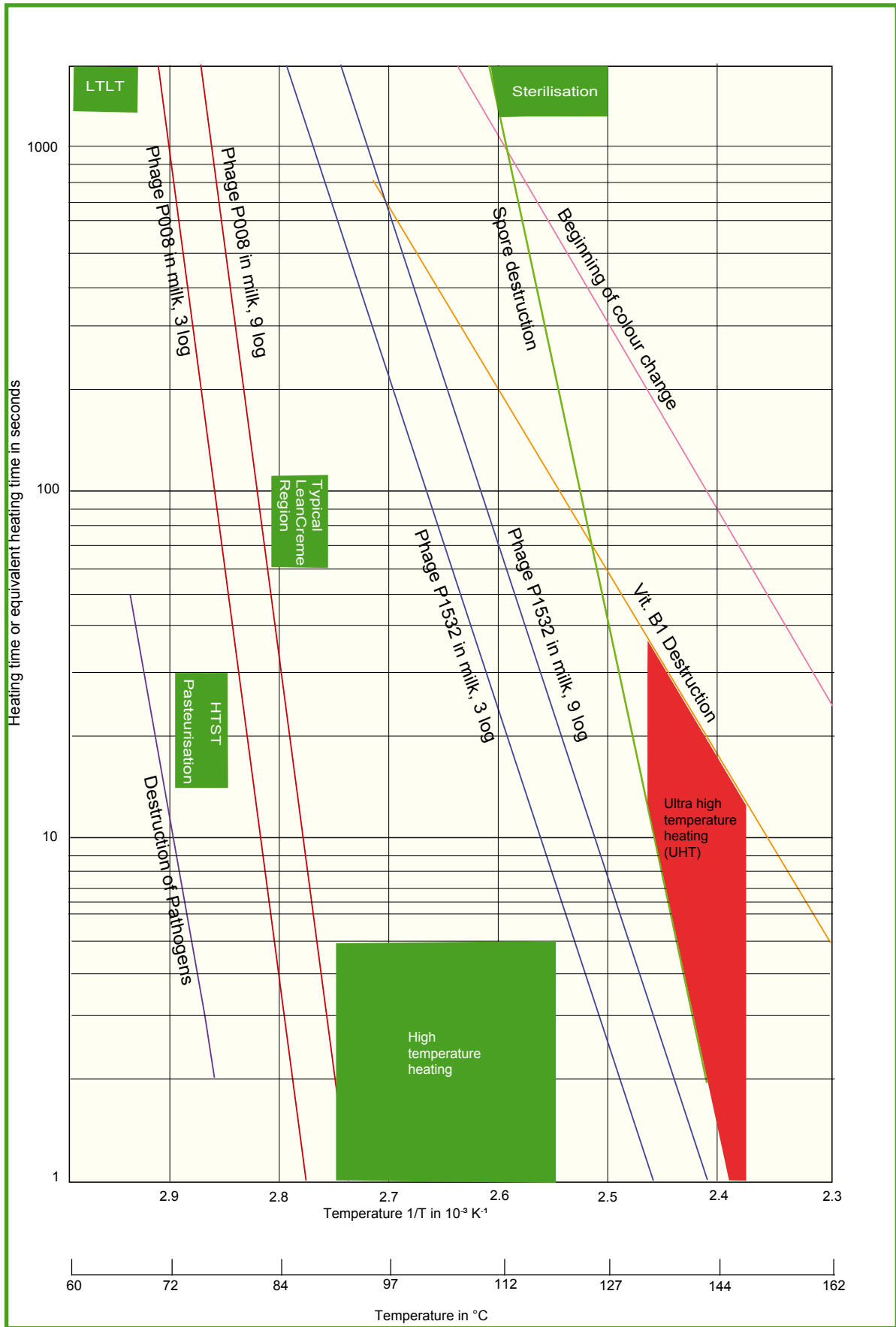


Fig. 7. Temperature time graphs of milk indicating lines of equal effect including inactivation of the most common bacteriophage P008, the extremely rare and heat resistant bacteriophage P1532. Region of typical LeanCreme temperature and time combinations are showed. (Kessler, 2002 and Atamer et al., 2009, modified)

As can be seen from fig. 7, the P008 phage are killed with a high log reduction under these heat treatment conditions. This complies fully with practical experience as SPX Flow Technology has no recorded cases of bacteriophage issues from microparticulated whey protein produced using APV LeanCreme™ technology.

It is not recommended to apply higher combination temperature/time of denaturation as it is known that the application of higher temperatures with the same range of holding time creates a different more compact structure of the microparticulated whey to the extent that less is incorporated into the cheese matrix.

Higher temperature would dramatically and irreversibly alter the whey microparticles high level of functional properties are formed, e.g. high water binding ability, high creaminess perception and high level of incorporation in the dairy matrix (e.g. cheese, yoghurt, ice crème among others). This high functionality is indeed based on a loose and open structure enabling active sites of the protein to interact with the components in the milk (Spiegel, 1999).

In conclusion the optimal heat treatment and shear can create an extremely high quality of microparticulate can be produced to boost the sensory and structural quality of the end product, while at the same time preventing contamination of the most common bacteriophage encountered in dairy.

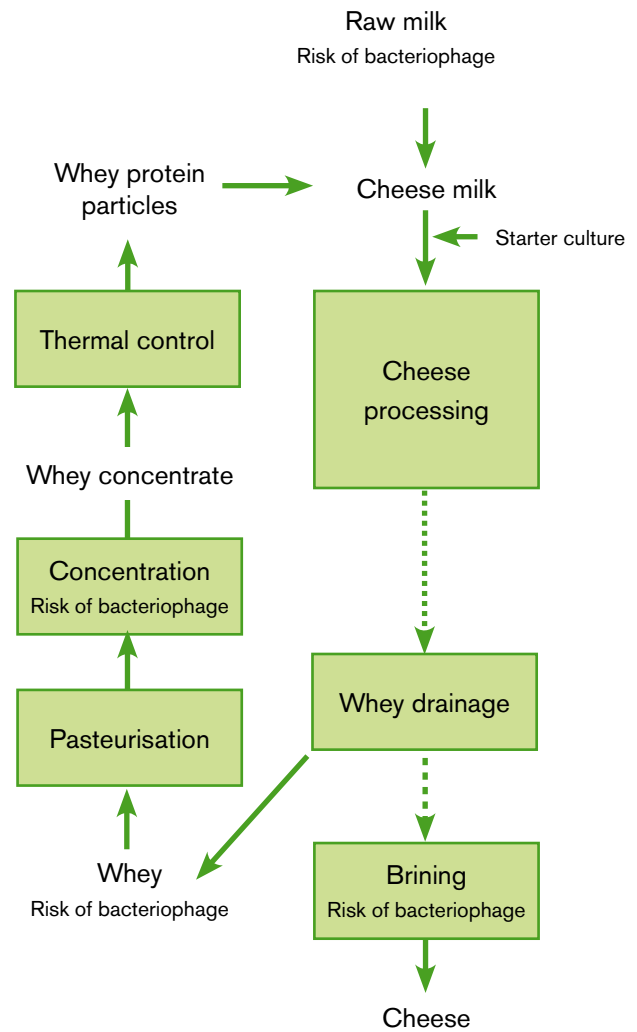


Fig. 8. Flow chart of a cheese process in which concentrated whey proteins are being reincorporated into the cheese milk thermal control of WPC is essential to avoid fermentation failures caused by bacteriophage.

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