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Experiences from *Listeria* consulting - Summary of the Colloquium of 29 August 2019

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Contents

Five speakers summarized the experiences of 30 years of *Listeria* consulting and prevention from different perspectives and showed what is possible today with modern techniques such as whole genome sequencing and MALDI-TOF.

1. 30 years of the *Listeria* Monitoring Program

René Imhof (Agroscope) looked back on **30 years of the *Listeria* Monitoring Program**. The beginning of the *Listeria* Monitoring Program dates back to the *Listeria* epidemic spanning from 1983 to 88 caused by Vacherin Mont d'Or cheese (122 cases led to 33 deaths; *Federal Office of Public Health, 1986; Bille, 1988; Bille, 1989; Büla et al., 1995*). Agroscope, or the Dairy Research Institute as it was called at that time, was commissioned to set up a laboratory for detecting *Listeria* and to define measures to rule out a repetition of such events.

Listeria are dangerous survivors. They outwit our immune system and survive inside the phagocytes of the immune system. In addition, they are opportunists, which in *Listeria monocytogenes* (*L. monocytogenes*) leads to special health risks for people with a weakened immune system as well as for pregnant women, younger people and older people, the so-called YOPI group (young [new born], old, pregnant or immuno-compromised people). Although other bacterial infections occur more frequently, the proportion of fatal disease progressions in listeriosis is particularly high.

Following the *Listeria* outbreaks in the 1980s, Agroscope implemented three measures:

1. A laboratory for *Listeria* analysis
In the beginning, the detection of *Listeria* took 65 days (1985), but in 1993, it became possible to detect *Listeria* in less than 10 days due to continuous improvements. Since 2006, it has been possible to detect *Listeria* in 48 hours.

2. The *Listeria* Monitoring Program (LMP)
The introduction of regular checks in two control loops (cheese dairies and commercial warehouses) ensured that no contaminated cheese was placed on the market, which also ensured cheese exports. The first phase, from 1990 to 2001, involved a great deal of effort and analysis to raise awareness and provide training, advice and assistance in the remediation of affected farms and to establish quality assurance measures in the farms. From 2002 to 2009, the effort was reduced, quality assurance was optimised and HACCPs were introduced. At the same time, Agroscope shifted its activities to advising and supporting regional consultants. Hygiene legislation was also adapted during this period, and the LMP was regionalised. The LMP in its old form has been obsolete since 2010 and has been reduced to a residual mandate.

3. The Agroscope *Listeria* Advisory Team
For *Listeria* problems, Agroscope's *Listeria* consulting team can be requested to assist and advise on operational remediation. The team consists of 3 – 4 members from different fields (manufacturing technology, hygiene and microbiology). Where necessary, the team can call in expertise or other experts from Agroscope's specialist areas. In the first step, the actual state is recorded (*Figure 1*) and the occurrence of *Listeria* in general and of *L. monocytogenes* in particular is investigated (environmental, smear water and product samples).

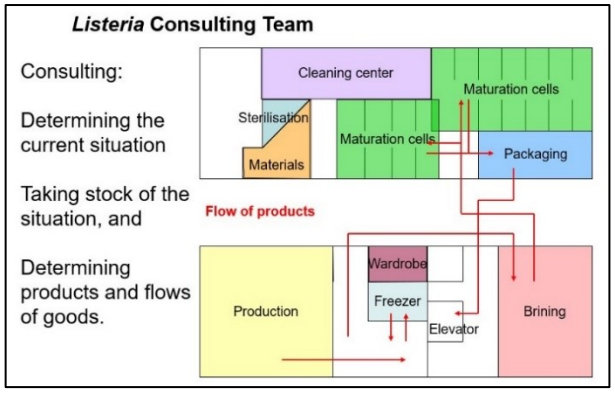

Figure 1. Determination of the actual state in the Listeria consultation

The product and goods flows are determined and the findings are incorporated into a risk and weak-point analysis (*Figure 2*), which then leads to restructuring proposals in the third step (*Figure 3*).

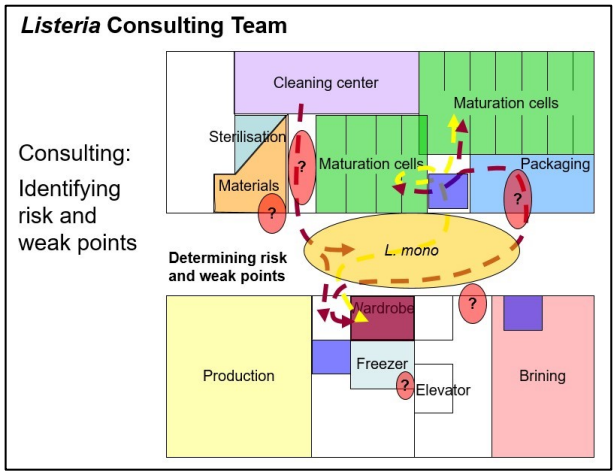

Figure 2. Description of the risks and weak points in Listeria consulting

Special attention is also paid to the boards for cheese ripening. They must be thermally treated with steam, which is equivalent to pasteurisation. Experiences from the LMP were consulted and outlines for company renovations were later miniaturised for alpine dairies, which can construct a simple steam treatment kit themselves (*Figure 4*). These instructions can be found in [Imhof & Riva Scettrini, 2015].

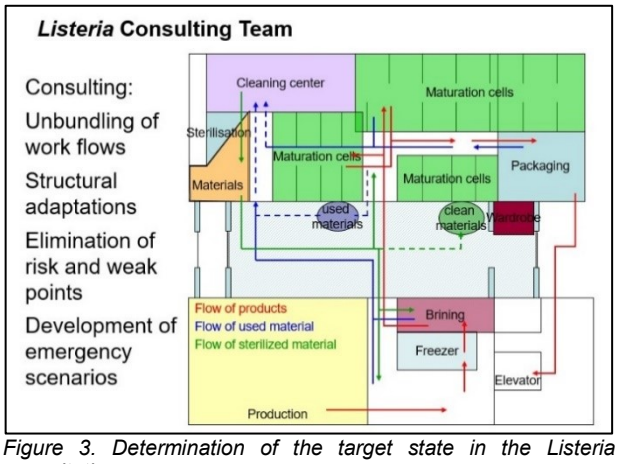

Figure 3. Determination of the target state in the Listeria consultation

Figure 4. Modular steam cell for Alps and small businesses

While the use of wood in cheese ripening is a controversial issue internationally, the effectiveness of steam treatment has been demonstrated and published in an experiment (*Imhof et al. 2017*; *International Dairy Federation, 2016*). This refutes the critical argument about whether wood can be hygienically prepared for use in contact with food by heat treatment in a steam chamber.

A research group at Agroscope is working on protective *Listeria* cultures. The LMP and consulting management have no direct experience with protective cultures or phage-based products during remediations in escalated situations. In second-hand cases, these products have not yet shown any sustained effect in acute *Listeria* situations. Customers are therefore not encouraged to use such products until their homework has been done. The effectiveness of such products in preventive use has yet to be proven.

A successful hygiene strategy is based on the following factors:

- having a disciplined QM system within the company
- applying the batch principle
- raising awareness and training staff
- using good manufacturing practice, HACCP and the hygiene concept

2. *Listeria* prevention in industry guidelines

Ernst Jakob (Agroscope) showed how successful ***Listeria* prevention in industry guidelines** could be implemented. A look at the figures in the European Rapid Alert System for Food and Feed (RASFF) shows where the problems lie with *L. monocytogenes* and cheese. The RASFF notifications on *L. monocytogenes* in cheese from the years 2009 – 2019 show bacterial counts between approx. 0.1 – 1,800,000 colony forming units (CFU)/g, of which, 44% had more than 100 CFU/g of *L. monocytogenes* (*Figure 5*).

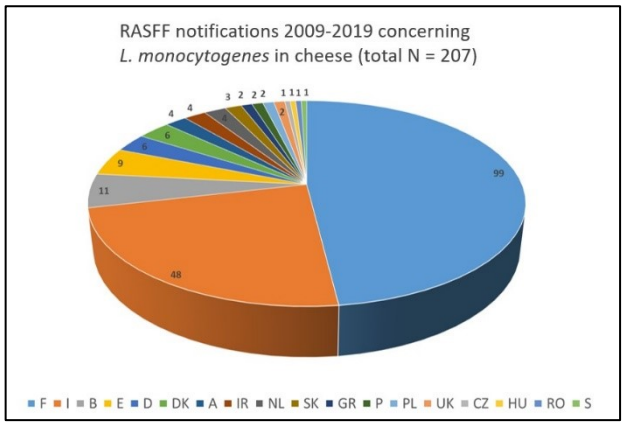

Figure 5. RASFF notifications 2009 – 2019 concerning L. monocytogenes in cheese, sorted by country

Most notifications came from France, Italy and Belgium; none comes from Switzerland. *Figure 6* shows that soft cheese is most likely to be affected in terms of firmness, although in many cases the cheese type was not specified in more detail.

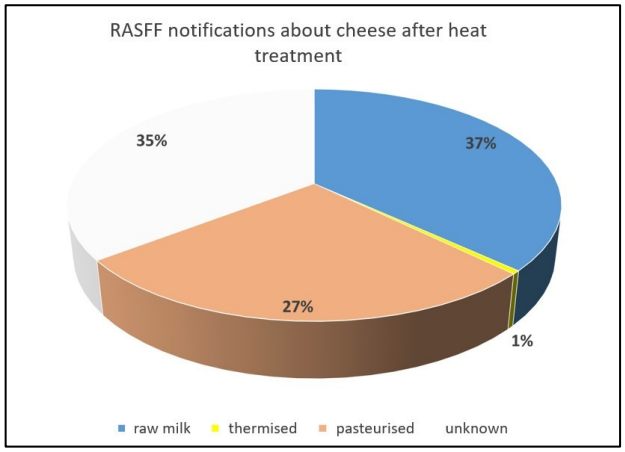

Figure 6. RASFF notifications 2009 – 2019 concerning L. monocytogenes in cheese, sorted by heat treatment

Figure 7 and *Table 1* also provide important information about the effective implementation of *Listeria* prevention. Contrary to popular belief, raw milk cheese is not the only critical product; very often pasteurised milk cheese is also a critical product, as *Listeria* problems are often due to recontamination. Cheeses matured for a long time are very safe products, as the probability of finding *Listeria* in them is low (*Federal Food Safety and Veterinary Office, 2017*).

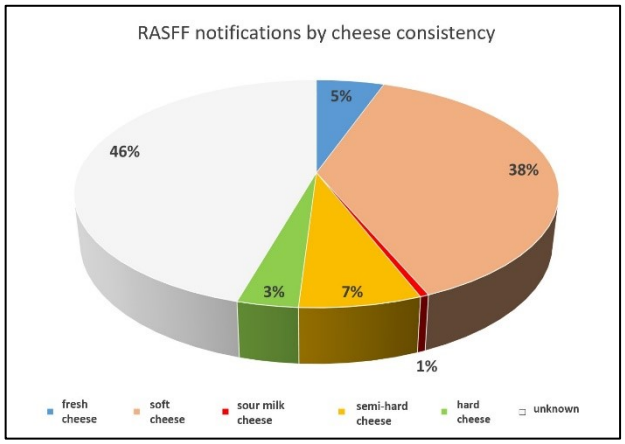

Figure 7. RASFF notifications 2009–2019 concerning L. monocytogenes in cheese, sorted by cheese type (cheese consistency).

Table 1. L. monocytogenes in hard cheese made from pasteurised milk (according to the evaluation of the National Research Programmes in the EU, Community Summary Report 2008 of the EFSA; A. Lutz, BLV, 2010)

Country	Sampling unit	Details	Units Tested Presence	*L. m.* presence in 25 g	Units Tested Enumeration	> detection =< 100 cfu/g	*L. m.* > 100 cfu/g
			N	% Pos	N	%	%
Cheeses made from milk from cows							
Bulgaria	Batch	at retail	2,502	0	231	0	0
Czech Republic	Batch	at processing	3,523	1.7	2,153	0	0
Germany	Single	at processing	682	1.3	214	0	0
	Single	at retail	3,172	0.5	1,621	0.1	0.2

year		milk	dough	rind	cause of error
2003	semi-hard cheese	raw milk	pos	pos	one producer supplied contaminated milk every day
2005	soft cheese with white mould	pasteurised	?	pos	cross-contamination with externally matured red smear cheeses
2006	semi-hard cheese	partly thermised (evening milk)	neg	pos	lubricating machine
2007	semi-hard cheese (alp)	raw milk	pos	pos	contaminated milk (10 - 100 CFU/ml)
2007	Raclette	pasteurised	neg	pos	water leakage from the sewer system into the cellar after flooding
2008	soft cheese	thermised	pos	pos	udder listeriosis of a cow, thermisation at 65 °C without heat holding time
2008	semi-hard cheese	raw milk	?	pos	trade cellar, source of contamination unknown
2008	semi-hard cheese	thermised	neg	pos	unknown, parallel production of Emmenthaler?
2008	semi-hard cheese	raw milk	pos	pos	unknown
2009	soft sheep cheese type Brie	pasteurised	?	pos	Listeria in Emmenthaler salt bath, cross-contamination
2009	semi-hard cheese	raw milk	?	pos	milk partially pos → contaminated milking aggregate (vacuum hose) at a milk producer
2009	soft cheese	thermised	pos	pos	supplier milk (mobile milking parlour, cistern water)
2009	semi-hard cheese (alp)	raw milk	pos	pos	unknown (dough pos < 10 CFU/g, lubricating water pos, salt bath pos)
2009	semi-hard cheese	thermised	neg	pos	unknown (salt bath neg)

Figure 9. Agroscope consultations 2003 – 2009, significance of process errors and cross-contaminations

Monitoring the environment is prescribed by law in accordance with Article 69 of the Hygiene Ordinance 'Sampling in processing areas and equipment' (*HyV, 2018*):

[2] Food establishments producing ready-to-eat foodstuffs, which could present a risk to human health caused by *Listeria monocytogenes*, must, within the framework of their sampling plan, test samples from the processing areas and the equipment used for *Listeria monocytogenes*.

- European Guide for Good Hygiene Practices in the production of artisanal cheese and dairy products (FACE, Farmhouse and Artisan Cheese & Dairy Producers Network) – 2016 (*Albrecht-Seidel, 2016*)
- QM Fromarte (artisanal dairies) – 2008 (*Anonymous, 2008*)
- SAV Leitlinie (summer pasture holdings) – 2015 (*Jakob & Menéndez Gonzàlez, 2015*)

Lebensmittel und Gebrauchsgegenstände **817.024.1**

Lebensmittelkategorie		Mikroorganismen/deren Toxine, Metaboliten	Probenahmeplan n	c	Grenzwert m	M	Analytische Referenzmethode	Stufe, für die das Kriterium gilt
1.2	Andere als für Säuglinge oder für besondere medizinische Zwecke bestimmte, genussfertige Lebensmittel, die die Vermehrung von *L. monocytogenes* begünstigen können	*Listeria monocytogenes*	5	0	100 KBE/g[18]		EN/ISO 11290-2[19]	In Verkehr gebrachte Erzeugnisse während der Haltbarkeitsdauer
			5	0	In 25 g nicht nachweisbar[20]		EN/ISO 11290-1	Bevor das Lebensmittel die unmittelbare Kontrolle der verantwortlichen Person des Herstellerbetriebs verlassen hat
1.3	Andere als für Säuglinge oder für besondere medizinische Zwecke bestimmte, genussfertige Lebensmittel, die die Vermehrung von *L. monocytogenes* nicht begünstigen können[21 22]	*Listeria monocytogenes*	5	0	100 KBE/g		EN/ISO 11290-2[23]	In Verkehr gebrachte Erzeugnisse während der Haltbarkeitsdauer

18 Die verantwortliche Person muss zur Zufriedenheit der zuständigen Vollzugsbehörde nachweisen können, dass das Erzeugnis während der gesamten Haltbarkeitsdauer den Wert von 100 KBE/g nicht übersteigt.
19 1 ml Inoculum wird auf eine Petrischale (140 mm Durchmesser) oder auf 3 Petrischalen (je 90 mm Durchmesser) aufgebracht.
20 Dieses Kriterium gilt für Erzeugnisse, bevor sie die unmittelbare Kontrolle der verantwortlichen Person des Herstellerbetriebs verlassen, wenn diese nicht zur Zufriedenheit der zuständigen Vollzugsbehörde nachweisen kann, dass das Erzeugnis den Grenzwert von 100 KBE/g während der gesamten Haltbarkeitsdauer nicht überschreitet.

22 Erzeugnisse mit einem pH-Wert von ≤ 4,4 oder a_w-Wert von ≤ 0,92, Erzeugnisse mit einem pH-Wert von ≤ 5,0 und a_w-Wert von ≤ 0,94; Erzeugnisse mit einer Haltbarkeitsdauer von weniger als 5 Tagen werden automatisch dieser Kategorie zugeordnet. Andere Lebensmittelkategorien können vorbehaltlich einer wissenschaftlichen Begründung ebenfalls zu dieser Kategorie zählen.

Figure 8. Legal requirements for L. monocytogenes (HyV, 2018)

The two legal limit values (*Figure 8*) of 'not detectable in 25 g' and '≤ 100 CFU/g' for those products in which *L. monocytogenes* cannot reproduce during the entire shelf life must also be observed, as proven by challenge tests.

Further helpful information is provided by the experience gained during the consulting process. *Figure 9* lists some consulting cases from the years 2003 – 2009. The marked cases show the serious influence of process errors (lack of holding time during thermisation) and crosscontamination, here by a contaminated salt bath.

The following industry guidelines are currently available in accordance with Regulation (EC) No. 852/2004 (*Regulation (EC) No. 852/2004, 2009*) and Article 80 of the Foodstuffs and Utility Articles Ordinance (*LGV, 2019*):

All industry guidelines are HACCP based. The measures listed in Table 2 belong to risk control.

Table 2. Important control measures for cheese-producing companies

What	How
Validated recipes	Validation as part of a HACCP study
Minimize entry via raw materials	Raw material monitoring Cold storage of raw milk Heat treatment etc.
Prevent entry from environment	Hygiene zones Good manufacturing practice Environment monitoring
Survival, minimizing growth	Recipe (firing temperature, maturing time etc.) Process monitoring Process hygiene controls
Final product	Process hygiene controls End product controls Dating

The production conditions according to the specifications for cheeses with 'Protected Designation of Origin' (PDO) (status as of 28.02.2018) must also be observed. Here, however, there are also requirements that lead to an increase in the risk of *Listeria*, for example for Vacherin fribourgois in milk storage conditions of up to 18 °C up to a maximum of 24 hours and optional thermisation (*Table 3*). Food safety has to be guaranteed, of course, which is not always easy.

Table 3. Selection of requirements for cheeses with 'Protected Designation of Origin' (PDO) according to their specifications (as of 28.02.2018). Some are critical with regard to Listeria prevention.

type of cheese	thermisation	scalding temperatur	milk storage		minimum maturing time
Berner Alpkäse	not allowed	≥ 50 °C	n.s. (18 °C)	≤ 15 h	4.5 months
Emmentaler	not allowed	52 – 54 °C	n.s. (18 °C)	≤ 24 h	4 months
Etivaz	not allowed	≤ 57 °C	max. 18 °C[2]	≤ 18 h	135 days
Formaggio d'alpe ticinese	not allowed	41 – 50 °C	n.s. (18 °C)	≤ 18 h	60 days
Glarner Alpkäse	not allowed	44 – 47 °C	< 13 °C	≤ 24 h	60 days
Gruyère	not allowed	54 – 57 °C	12 bis 18 °C	≤ 18 h	5 months
Sbrinz	not allowed	54 – 57 °C	n.s. (18 °C)	≤ 24 h	18 months
Tête de Moine	not allowed	44 – 53 °C	≤ 18 °C[3]	≤ 18 h[3]	75 days
Vacherin fribourgeois	optional (ALP pos.)[1]	30 – 36 °C	n.s. (18 °C)	≤ 24 h	70 days
Vacherin Mont d'Or	57 to 68 °C ≤ 15s (ALP pos.)	32 – 38 °C	10 – 18 °C	≤ 20 h	17 days
Walliser Raclette	not allowed	36 – 45 °C	< 8 °C[4]	≤ 24 h	3 months
Werdenberger/Liechtensteiner Bloderkäse	can: 55 – 69 °C/≥ 15s (ALP pos.)	≤ 45 °C / pH < 4.65	n.s. (18 °C)	≤ 24 h	none
Werdenberger/Liechtensteiner Sauerkäse	can: 55 – 69 °C//≥ 15s (ALP pos.)	≤ 45 °C / pH < 4.65	n.s. (18 °C)	≤ 24 h	2 months

s.p. = not specified, i.e. the legal requirements apply (max. 18 °C)
ALP pos. = alkaline phosphatase reaction must be positive after treatment
[1] temperature and time are not defined
[2] maximum temperature of stored evening milk in the morning
[3] if the milk is cooled below 8 °C, it may be stored for a maximum of 24 hours
[4] summer pasture holdings may store milk at < 13 °C

In addition to the factors mentioned above, the quality of the raw materials is also decisive for the manufacture of safe products. In contrast to other countries (*Table 4*), where the prevalence of *Listeria* in milk is up to almost 20%, the raw milk used in Switzerland from cows not fed silage is decisive and is only 0.33% (*Imhof, 2014*).

Table 4. Prevalence of L. monocytogenes in raw milk (Kuousta M. et al., European Dairy Magazine, No. 2, 2010)

Sample	Total samples	Prevalence %	Country (Publ. Year)
Farm bulk milk	294	1.0	Sweden (2002)
Dairy silo milk	295	19.5	Sweden (2002)
Farm bulk milk	861	6.5	USA (2004)
Farm bulk milk	113	5.3	Northern Ireland (1992)
Dairy silo milk	113	33.3	Northern Ireland (1992)
Farm bulk milk	948	5.9	USA (2003)
Farm bulk milk	774	3.6	Spain (1998)
Farm bulk milk	131	4.6	USA (2001)
Farm bulk milk	1720	2.7	Canada (1997)
Farm bulk milk	589	7.9	Irland (1992)

Other critical factors are the growth of *L. monocytogenes* in milk as a function of storage temperature and time (*Figure 10*) and the influence of heat treatment on germ reduction as a function of temperature at a holding time of 15 s (*Table 5*).

The maturing time of raw milk cheese also has an influence on the survival of *L. monocytogenes*. Bachmann and Spahr were able to show in 1995 that *L. monocytogenes* was no longer detectable in hard cheese after only one day, whereas in semi-hard cheese, even after 90 days, *L. monocytogenes* was only reduced to about one tenth of the original bacterial count.

The examples in *Figures 11 and 12* are exemplary with regard to which requirements regarding *L. monocytogenes* must be derived from current knowledge in order to be able to control the *Listeria* risk.

In summary, it can be stated that the Swiss industry guidelines for the dairy industry (QM Fromarte and SAV Guidelines):

- are HACCP-based
- interpret the legal requirements in a pragmatic yet scientifically sound manner
- are certifiable in the case of QM Fromarte (implementation control through regular audits)
- where necessary, support farms in the implementation of the guidelines by the dairy consultants of the regional advisory platforms

As a result, there are very few recalls of Swiss cheese due to *Listeria*.

The Swiss experiences have been summarised together with the German and Austrian experiences in the Inter-Lab Guide to *Listeria* in Milk Products (*Figure 13*). In it, the authors commit themselves to *Listeria*-free cheese production (no use of the limit value of ≤ 100 CFU/g). The guideline (*InterLab, 2018*) and an overview article (*Becker, 2017*) can be found under the following links:

- Guideline, Website Agroscope, Liebefeld
- Guideline, Website HBLFA Tirol, Rotholz
- Guideline, Website MUVA, Kempten
- Overview article, B&L Medien Gesellschaft, Hilden

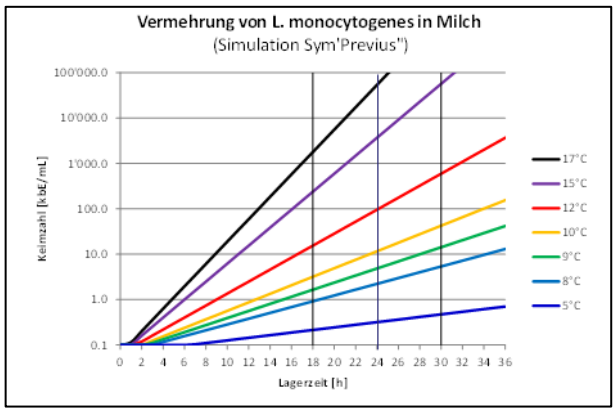

Figure 10. Propagation of L. monocytogenes in milk at different temperatures, calculated with Sym'Previus

Table 5. Influence of heat treatment on bacterial reduction of L. monocytogenes in milk (Sörqvist, 2003)

Thermisation conditions		Germ reduction	
Temperature	Holding time	*Listeria monocytogenes*[1]	*Salmonella* spp.[2]
57 °C	15 s	< 0.1 log	0.2 log
62 °C	15 s	0.2 log	1.5 log
65 °C	15 s	0.7 log	5.7 log
68 °C	15 s	2.0 log	> 7 log

[1] Calculation basis: D value at 65 °C in milk: 21.6 s, z value: 6.7 °C (Sörqvist, 2003)
[2] Calculation basis: D value at 65 °C in various media: 2.6 s, z value: 5.2 °C for *Salmonella* spp. without *S. senftenberg* (Sörqvist, 2003)

Hard cheese, thermised 65 °C/15 s (or at least equivalent)

HACCP Plan

oPRP	Criterion	Sample material	Frequency*	Specification
oPRP5 (salting)	*Listeria* spp.	Brine (stabilised with $CaCO_3$ if necessary)	8 x per year or 1/180 batches	n.d. / 25 g,ml
oPRP6 (maturation)	*Listeria* spp.	Lubricating water after cheese care (for red smear cheese) or rind sample (scraping)	8 x per year or 1/45 batches	n.d. / 25 g

* *Minimum frequency (applies only if no listeria has been detected in a series of n=5 samples)*

Figure 11. HACCP plan for L. monocytogenes for semi-hard cheese in the factory, revision 2019 of the QM Fromarte
oPR = operational prerequisite programs; n.d. = not detectable

Semi-hard cheese, thermised 65 °C/15 s (or min. equivalent)

Request in retail (end best-before date)

Criterion	Sample material	Specification
Listeria monocytogenes	edible part, if necessary with rind part	n.d. / 25 g,ml

Figure 12. HACCP plan concerning L. monocytogenes for semi-hard cheese on the market, revision 2019 of the QM Fromarte
n.d. = not detectable

3. Consulting experience from the point of view of an industrial company

Thomas Stanke (Sachsenmilch) reported on his **Consulting experience from the point of view of an industrial company**. In his impressive presentation, Thomas Stanke showed that the experiences of *Listeria* consulting could be transferred from alpine operations to industrial processing in village cheese dairies and also to large industrial companies. The *Listeria* consulting team is purely preventive and has the task of nipping any blind spots in the bud.

In addition to the usual measures to avoid *Listeria* problems, the most sensitive analytics are used in large-scale operations (*Figure 14*) to detect and eliminate residues of *Listeria* subspecies in ambient samples. Where necessary, structural measures are taken immediately. This makes it possible to produce *Listeria*-free cheese even in large quantities and to assert oneself on the market.

QUALITÄTSMANAGEMENT

InterLab Leitfaden

Listerien in Milchprodukten

InterLab dankt der Fa. Funke-Dr. N. Gerber Labortechnik für die Unterstützung!

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[3] HBLFA Tirol, Standort Forschung und Service Rotholz (Österreich)
[4] MUVA Kempten GmbH (Deutschland)

Der Krankheitserreger *Listeria (L.) monocytogenes* stellt nach wie vor eine große Herausforderung für Käsereien dar. Listerien sind eine der häufigsten Ursachen von Rückholaktionen aus mikrobiologischen Gründen bei Lebensmitteln. Abgesehen von möglichen schweren Erkrankungen beim Menschen können daher Kontaminationen der Lebensmittel mit *L. monocytogenes* zu großen wirtschaftlichen Verlusten für den Lebensmittelunternehmer führen. Das Vorhandensein von zwei unterschiedlichen Grenzwerten (nicht nachweisbar in 25 g versus 100 koloniebildende Einheiten pro Gramm) macht es zudem schwieriger zu entscheiden, wie mit positiven Ergebnissen umgegangen werden soll.

Aus diesem Grund hat die Internationale Gemeinschaft der Laboratoriumsleiter in der Lebensmittelwirtschaft (InterLab) einen Leitfaden für Listerien in Milchprodukten ausgearbeitet [9]. Der Leitfaden gibt Empfehlungen zur Umsetzung der Vorgaben der Verordnung (EG) Nr. 2073/2005 [8] über mikrobiologische Kriterien für Lebensmittel und zur Beurteilung der Verkehrsfähigkeit gemäß Verordnung (EG) Nr. 178/2002 (Basisverordnung) [8]. Der Leitfaden dient als Hilfsmittel für milchwirtschaftliche Be-

Figure 13. Overview article on the InterLab Guide to Listeria in Milk Products (Becker, 2017)

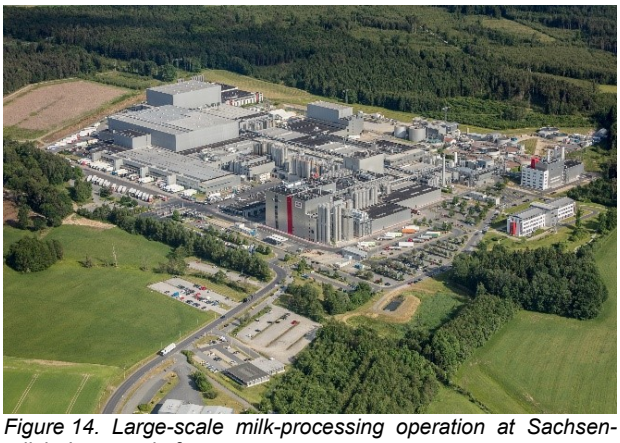

Figure 14. Large-scale milk-processing operation at Sachsenmilch, Leppersdorf

4. Potential of whole genome sequencing

Lena Fritsch (Anses) provided information on the **Potential of whole genome sequencing**. This relatively new technique currently has the highest discriminatory capacity and enables the identification of new correlations (*Figure 15*).

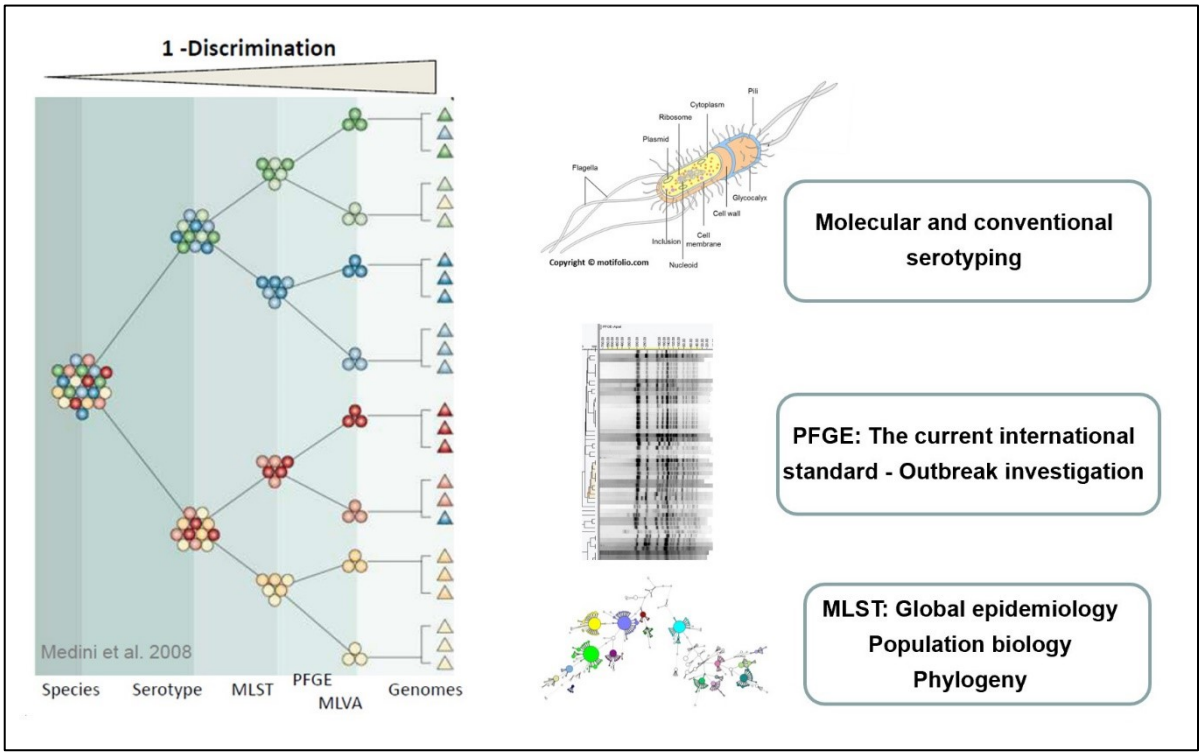

Figure 15. Overview of available typing techniques for L. monocytogenes

In whole genome sequencing (WGS), the bacterium to be examined is taken from a pure culture; the DNA is extracted and then sequenced. The resulting 'reads' are then assembled, that is, they are reassembled into the now-known genome. Any genomic variants can also be identified in comparison to the reference genome (*Figure 16*).

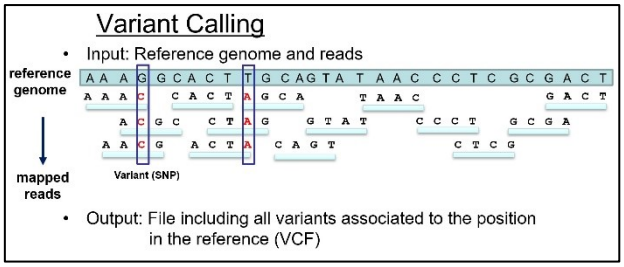

Figure 16. Identification of genomic variants by comparing the reads with a reference genome

The genetic information obtained in this way can be used, for example, to clarify outbreaks, identify genetic markers, carry out risk assessments and identify outbreak sources.

The introduction of WGS has already led to a situation where, in monitoring listeriosis

- the outbreak sources are identified more frequently and quickly (see also *Figure 17*); and
- the total number of outbreak-associated cases identified has increased.

The WGS provides food manufacturers

- a fast and efficient clarification of the causes of contamination (e.g. ingredients), and
- a distinction between new and recurring (persistent) contaminations (*Jackson, 2016*).

However, the SNP (single nucleotide polymorphism, the name for a variation in a single base pair in a DNA double strand) distances, the tree topology, the inclusion of epidemiological data and information on traceability must be taken into account. This is important information because

- different bacterial populations have different levels of genetic diversity,
- of genetic drift, natural selection, etc.,
- isolation techniques and cultural characteristics

also play an important role and, if ignored, can lead to false-positive findings. *Figure 18* gives an example.

There is still room for improvement in the use of the WGS in outbreak investigations. These improvements concern:

- the relationships between WGS and the typing data used so far (serotype, clonal complex CC)
- information on discrimination between findings (requires bioinformatics skills)
- the dissemination of WGS information and the regulation of the rights thereto (e.g. database access rights)
- long-term data storage
- the standardization of technology

So far, little research has been done into genetic markers, WGS-based risk assessment and allocation to outbreak sources. In order to identify genetic markers, genome-wide association studies are required. These could help to identify phenotypic differences between strains (e.g. regarding persistence, antibiotic resistance, virulence or stress tolerance). This information could lead to improved hazard identification, adjustments in the management of food safety risks, improvements in predictive food microbiology and improvements in risk assessment (more focus on high-risk sub-populations of pathogenic organisms).

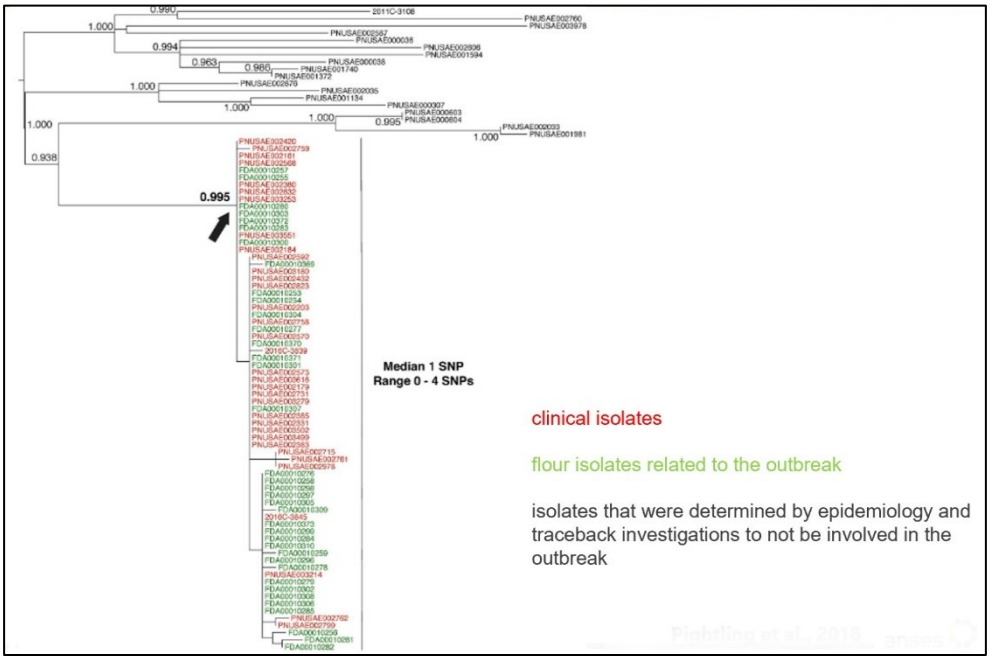

Figure 17. Comparison of Listeria sequences from clinical and flour samples in the clarification of an outbreak (Pightling, 2018)

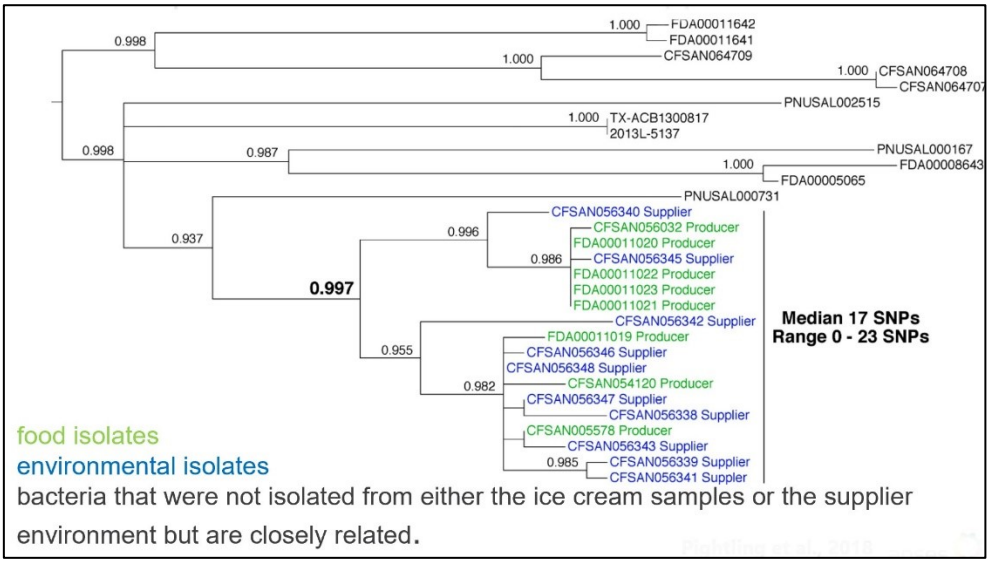

Figure 18. Example of false-positive source identification of L. monocytogenes isolates from an ice cream sample and environmental samples from a supplier (Pightling, 2018)

When assigning strains to outbreak sources, it can be seen that the highest exposure of almost 52% concerns the least virulent strains, while contact with very virulent strains occurs in less than 10% of cases. The virulent strains are distributed differently among the different foods (*Figure 19*).

In summary, it can be said that WGS

- enables the 'all in one' approach;
- has strong potential for discrimination, which is an added value in outbreak investigations;
- allows promising new applications;
- still has some gaps in the harmonisation of quality criteria and standardisation;
- still has questions to be answered when making the data available;
- is recognised and used by the European Food Safety Authority EFSA.

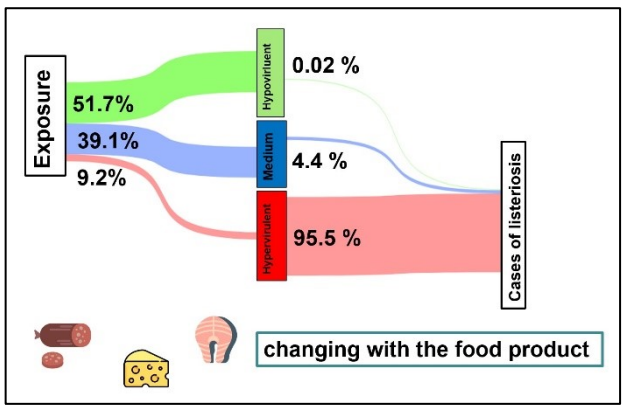

Figure 19. Exposure rate and virulence in listeriosis cases, changes depending on food

The WGS thus offers promising opportunities but is of limited benefit to those without a biological context and those in public health.

5. Potential of MALDI-TOF

Alicia Romanó (Agroscope) reported on the **Potential of MALDI-TOF** in her presentation. The acronym MALDI-TOF stands for matrix-assisted laser desorption ionization-time of flight and is a method for the ionization of molecules and for their mass spectrometric analysis. The technique is not new; first data on bacterial investigations were presented several years ago. However, further developments have now made the technique a valuable tool, as can be seen, for example, from the number of publications in which it appears. There are currently more than 100 publications with the keywords 'MALDI-TOF' and 'public health'.

During the examination of samples, a small amount of one pure colony is applied on a target, mixed with a special matrix, dried and precisely heated and vaporized with a laser. It is essential to pay attention to the sample quantity (*Bastin, 2018*). In the MALDI process, a UV laser is used to transform the protein and peptides of isolated micro-organisms into positively charged ions. The matrix absorbs the laser energy and transfers protons to the intact proteins in the gas phase. The ions are then accelerated electronically and reach the flying tube at a mass-dependent speed. Since the different proteins and peptides have different masses, the ions reach the detector at different times (= flight time, time-of-flight: TOF). The device measures the time between the pulsed acceleration and the corresponding detector signal of the ions, and then the time is converted into precise molecular masses. The characteristic spectra (proteomic fingerprints) are compared with reference spectra (*Figures 20* and *21*).

The advantages of the method are:
- rapid results
- low running costs
- good repeatability
- robust and reliable results

Critical issues include:
- the sample quantity
- the initial costs
- the regular extension of the reference spectra library

At Agroscope, MALDI-TOF mass spectrometry is used for identifying lactic acid bacteria and yeasts, as well as for experiments for differentiating strains in starter cultures, for example *Streptococcus thermophilus*.

Alicia Romanó also uses the method in her dissertation. The goal of her research is to study whether there is a correlation between the resistoma found in the bovine mammary gland and the bacteria isolated by environmental samples. The intramammary resistoma are related to the environment (litter, liner, teat surface) (see also *Figure 22*).

For more than 1600 samples, the use of MALDI-TOF is the easiest and fastest way to identify the species contained in the samples. The samples are enriched on blood or selective agar and the pure cultures are identified with MALDI-TOF. So far, 1484 different strains belonging to 102 different species could be determined from 1200 samples, 64 % of which were *Staphylococcus* subspecies.

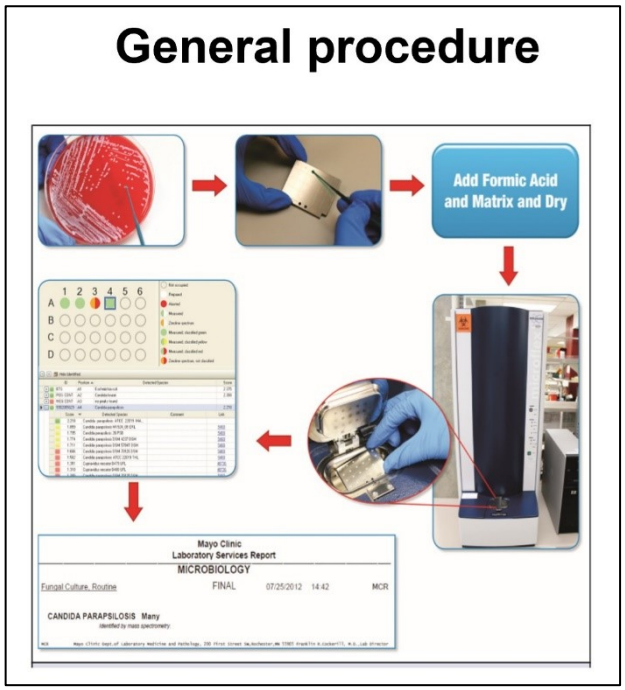

Figure 20. Procedure for MALDI-TOF analysis

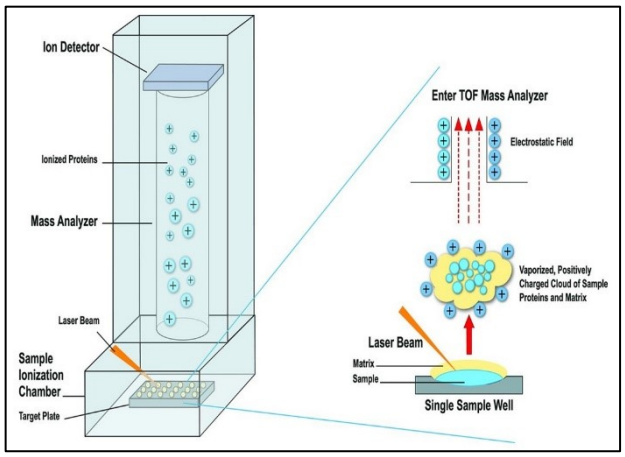

Figure 21. How MALDI-TOF works in the investigation of infectious diseases (Patel, 2015)

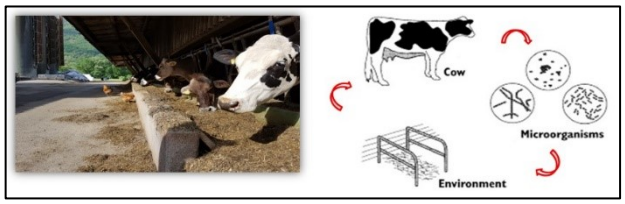

Figure 22. Relation between the intramammary resistoma and its environment (bedding, liner, teat surface)

Further examples of contexts in which MALDI-TOF can be successfully used include:
- water analysis of environmental samples to confirm *Legionella* findings
- pharmaceutical microbiology
- taxonomic research
- food microbiology (confirmatory analysis of positive findings of bacteria, *Cronobacter*, *Salmonella* spp.)
- veterinary bacteriology (mastitis pathogens, unusual species)
- subtyping of antibiotic-resistant germs such as KPC-producing *Enterobactericeae* or MRSA in *Staphylococcus aureus*

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