Testing of the applicability of battery-powered portable microbial air samplers for detection and enumeration of airborne *Lactococcus lactis* dairy bacteriophages

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1. Introduction

Infection of lactic acid bacteria used as starter cultures in dairy factories by bacteriophages is still a major cause for deteriorating acidification processes during milk fermentation (1-3). Phages attacking *Lactococcus lactis* have been known since the 1930's, when Whitehead and Cox in New Zealand observed that phages were responsible for the failure in the acid producing activity of a cheese starter culture (4). Since then, phage control measurements have been key activities for preventing the dissemination of phages in the dairy factory. Success in phage control relies on a number of important practical approaches including appropriate sanitation (cleaning and desinfection), adequate ventilation (including air flow control) and in particular selection of suitable starter cultures revealing a reliable phage resistance phenotype (5). Phages can easily be disseminated in the dairy environment and phage monitoring is a prerequisite for assessing the specific phage risks in a dairy factory (6-13). Phage can accumulate rapidly in high numbers in whey, and all whey processing steps are critical with respect to phage control. Phages are easily spread, when whey contaminated by phages forms an aerosol. In particular, whey separators can be regarded as indoor bioaerosol nebulizers.

We have previously investigated the phage load of the indoor air in a dairy factory producing fresh cheese (14). Using an MD8 air sampler, we have established an optimised protocol for collecting *L. lactis* phages on gelatine membrane filters. We demonstrated a high number of airborne phages nearby the whey separator as the source of the phage bioaerosol and a drift of the phage bioaerosol into the production hall. The objective of the present communication was to compare the sampling and recovery efficiencies of two portable battery-operated air samplers with those obtained previously with the MD8 sampler. The sampling principles of the two portable devices were different and were based either on gelatine membrane filtration (AirPort MD8 unit) or on impaction on agar (MAS-100 device). Phages retrieved were not only enumerated by the conventional agar-overlay method but were also used in a starter activity test.

2. Material and Methods

2.1 Bacteria, phages and media

A mesophilic, undefined mixed-strain starter culture was used in the dairy continuously for the production of fresh cheese (6, 15, 16). Single-colony isolates obtained from the culture were grown in (lactose-containing) M17-broth (17) or in litmus reconstituted skim milk at 30°C. Cultures were stored frozen at –76°C for long-time maintenance or were

propagated biweekly and stored in between at 4°C. Phage-containing whey samples were filter-sterilized (0.45 µm pore size) and 20-µl aliquots were spotted onto bacterial lawns of culture isolates in order to select phage-sensitive indicator strains (14, 16).

2.2 Air sampling

Air samples were taken in close proximity to a running whey separator and at various distances from this phage aerosol source in the same room and in the next hall. Sampling was done during the production hours of the factory. Air samplers were running in 1-m height from the floor. The two portable air samplers (see 2.3 for details) were operated on different production days. The same reference MD8 apparatus (Sartorius, Göttingen, Germany) was used in both test series as a reference (14) and was operated at the same locations in approx. 1-m distance to the portable samplers. Data for phage recovery from aerosols presented in this communication are calculated from single samplings due to the limited time available for sampling during the production hours. It should further be noted that various activities of the staff personel during the samplings (i.e., walking, cleaning, opening of the door etc.) could not be avoided.

2.3 Microbiological air samplers

Sterile gelatine membrane filters (pore size: 3 µm, diameter: 80 mm) were used for sampling with the MD8 air sampler (14) and with the AirPort MD8 sampler (Sartorius, Göttingen, Germany). 100-l air samples were collected within 2 min, when both units were compared (unit setting: 3 m^3 per h [MD8] and 50 l per min [AirPort MD8]). Handling of filters after sampling was essentially done according to an optimised protocol described earlier (14). This protocol is designated as experimental set-up A throughout this communication. Filters were removed from the filter holder with sterile forceps and were transferred into sterile plastic bags (size: 10x15 cm) specified for a stomacher lab blender (Seward Laboratory, London, UK). 5 ml of ice-cold filter resuspension buffer (¼-strength Ringer´s solution / 10% [vol/vol] skim milk; [14]) were added to each filter. Bags were sealed and stored chilled on ice during transportation to the laboratory. The following handling steps were as follows: 2-min incubation in a 37°C water-bath, mechanical 2-min resuspension, serial dilutions in phage diluent (¼-strength Ringer´s solution / 10% [vol/vol] M17 broth [14]) and finally determination of plaque-forming units in duplicate with a phage-sensitive indicator strain (14).

For comparison of the MD8 air sampler with the MAS-100 sampler (Merck, Darmstadt, Germany), 100-l air samples were collected with both units within 1 min. Five different experimental set-ups were tested for the MAS-100 air sampler. Standard petri dishes (diameter: 90 mm) were used in all cases and were prepared before sampling as follows:

- *protocol B1:* M17 bottom agar with an overlay of 3 ml M17 soft (0.7%) agar
- *protocol B2*: M17 bottom agar with an overlay of 3 ml gelatine (15%)
- *protocol B3:* M17 bottom agar with an overlay of 3 ml M17 soft (0.7%) agar seeded with 0.3 ml of an overnight culture of indicator bacteria grown in M17-broth
- *protocol B4:* M17 bottom agar
- *protocol B5:* Gelatine membrane filter specified for MD8 and AirPort MD8 samplers

After sampling, petri dishes were kept chilled at 4°C during transportation to the laboratory and handled as follows:

The M17 soft agar was resuspended in 5 ml of phage resuspension buffer (protocol B1); 2 ml of the same buffer were added to the melted gelatine overlay to obtain a 5-ml final volume (protocol B2). The M17 soft agar samples were cleared in a table centrifuge (20 min, 1,500xg) before membrane filtration (0.45 µm pore size) of the supernatants. Plaque assays were done in duplicate with serial dilutions performed in phage diluent as described earlier (14). Plates from protocol B3 were incubated without further treatments at 30°C. 3 ml of M17 soft agar seeded with overnight indicator bacteria were poured on plates from protocol B4. Gelatine filters (protocol B5) were handled as described above.

Incubation of all plates was done overnight at 30°C. Phage-derived plaques were counted and phage titers were expressed as plaque-forming units (PFU) per m^3 indoor air. Results obtained with the MAS-100 sampler were statistically corrected according to the positive hole conversion table provided by the supplier which is taking into account the total number of holes (i.e., 400) of the sampler head (18). The essentials of the 5 MAS-100 experimental set-ups are summarized in Table 1.

Tab. 1: Experimental set-ups for collecting phage bioaerosols with the MAS-100 unit (see also text)

	Protocol				
	B1	**B2**	**B3**	**B4**	**B5**
M17 bottom agar	X	X	X	X	
M17 top agar layer (0.7%)	X				
Gelatine (15%) top layer		X			
M17 top agar (0.7%) seeded with indicator bacteria before sampling			X		
M17 top agar (0.7%) seeded with indicator bacteria after sampling				X	
Serial dilutions of resuspended top layer before plaque assay	X	X			
Direct incubation without serial dilutions			X	X	
Dry gelatine membrane filter					X
Serial dilutions of resuspended gelatine filter before plaque assay					X

2.4 Starter culture activity test

10 ml skim milk were inoculated 2% (vol/vol) with fresh over-night cultures of either the original starter culture or of single-colony isolates from the original culture. 0.2 ml of resuspended gelatine overlay from protocol B2 containing a high or a low number of collected phages were added to the tubes (final phage titer $9x10^4$ PFU/ml or $9x10^1$ PFU/ml, respectively). For the controls, aliquots were preheated for 20 min at 90°C for phage inactivation. Tubes were incubated at 30°C and the pH was recorded over time.

2.5 Transmission electron microscopy

Negative staining of phage samples with 2% uranyl acetate was done as described before (19-21). Micrographs were taken in a Tecnai 10 electron microscope (fei, Eindhoven, The Netherlands) at an acceleration voltage of 80 KV.

3. Results and Discussion

Analysis of phage population

Air samples were taken in a dairy plant using one single undefined mixed-strain starter culture continuously. The phage population persisting in the dairy throughout our investigation was analysed and consisted of small isometric-headed phages. The phage morphology is shown in Fig. 1. This phage type is a member of the 936 phage species which is one of the predominating *Lactococcus lactis* phage types throughout the world (22-23). 936-like phages have been characterized in detail, and the genome sequences of representatives of this phage species have been published (i.e., phages sk1 [24] and bIL170 [25], respectively). A representative of the factory-specific phages (i.e., phage P482) has also been sequenced recently (26).

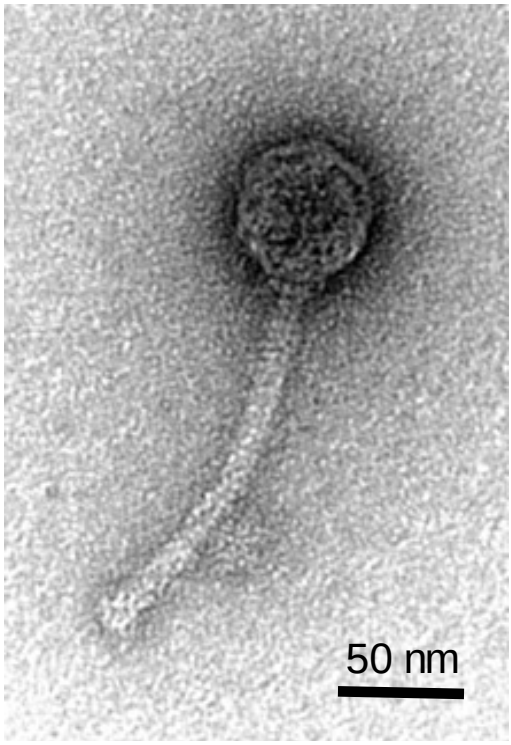

Fig. 1: Transmission electron micrograph of the *L. lactis* phage present in the indoor air of the dairy factory during air sampling.

Comparison of MD8 and AirPort MD8 air samplers

In a previous study, we reported on the dissemination of airborne *L. lactis* phages in the same dairy plant. Sampling was done with an MD8 apparatus, and phage numbers in the indoor air ranging from $2x10^8$ PFU/m^3 to $2x10^2$ PFU/m^3 were documented at various places and distances from the phage aerosol source (14). As an alternative, the small portable, battery-powered AirPort MD8 unit is available from the same supplier. The same gelatine membrane filters are used in both devices, and we were interested to compare the sampling efficiencies of both samplers (Fig. 2). Bioaerosol samples were taken in parallel with both units with identical settings next to the source of the phage aerosol (1-m distance on left and right side [A , B in Fig. 2]) and at various distances from this source (i.e., at 5 m, 10 m, 15 m [C-E, Fig. 2]). Fig. 2 illustrates that the sampling efficiencies of both apparatus were nearly the same. The decline of phage numbers in the air at the different locations from $5.2x10^6$ PFU/m^3 to $8.4x10^5$ PFU/m^3 (MD8 data) within the same hall was low, indicating that distribution of the phage aerosol within the hall was very efficient.

Comparison of MD8 and MAS-100 air samplers

The MAS-100 air sampler represents a battery-driven compact impactor type of samplers based on the principles originally described by Andersen (27). Standard petri dishes were placed in the collecting head under a 400-hole sieve plate. Collecting airborne viruses with impaction samplers is not a standard application (28-30). Therefore, we were interested to test the applicability of this apparatus for recovering airborne dairy phages.

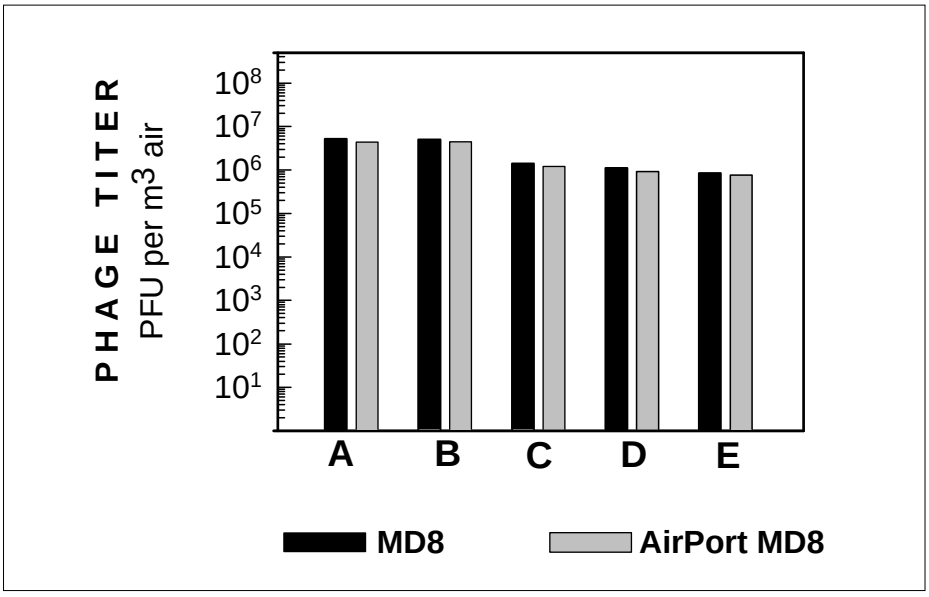

Fig. 2: Comparison of the MD8 (black bars) and the Airport MD8 (grey bars) air samplers for collecting *L. lactis* phages from whey-derived aerosols at different locations from the aerosol source (1 m [A & B], 5 m [C], 10 m [D], 15 m [E]).

The MD8 sampler was used as a reference sampler (14). Data from 3 sampling places are shown in Fig. 3 with varying phage counts in the ambient air (i.e., $5.2x10^7$, $9.7x10^6$ and $7.1x10^4$ PFU/m^3 as determined with the reference MD8 unit). Various sampling procedures were tested according to Table 1 (i.e., set-ups B1-B4). Sampling efficiencies were always lower for the MAS-100 apparatus. MAS-100 phage recovery rates were varying from 1% to 5% when compared to those obtained with the MD8 unit. Recovery rates were independent of the collection overlay medium (M17 soft agar or gelatin (see B1 and B2 bars in Fig. 3a-c). Fig. 3c (bars B3 and B4) illustrates that the time of adding indicator bacteria was not crucial (i.e., impaction of phages on a lawn of indicator bacteria embedded in M17 soft agar *versus* phage collection on M17 agar followed by subsequent floating of the plates with indicator bacteria in M17 soft agar in the laboratory).

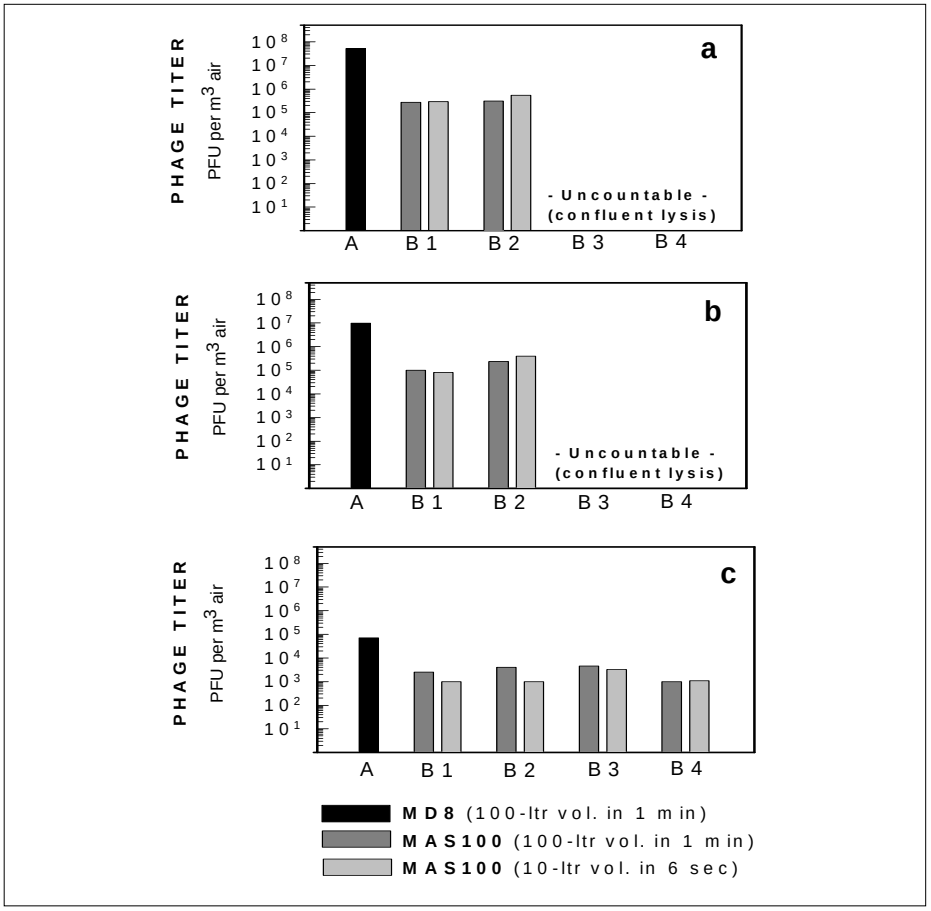

Fig. 3: Comparison of the MD8 (black bars) and the MAS-100 (grey bars) air samplers for collecting *L. lactis* phages from whey-derived aerosols close to the aerosol source (a) and at 2 locations in the next hall in front of the closed door (b) and at a 10-m distance from the door (c). The experimental set-ups used for the MD8 (A) and the MAS-100 unit (B1 - B4) are indicated in the text and in Table 1. Two sampling times were used for the MAS-100 apparatus (i.e., 1 min [dark grey bars; 100-l volume] and 6 sec [light grey bars, 10-l volume]).

Direct sampling and counting of phage-derived plaques without subsequent decimal dilution steps was only practicable from bioaerosols with a relatively low phage load (i.e. < $1x10^5$ PFU/ml; MD8 data) and was not possible at higher phage counts, since plates were overloaded. This technical limitation was still present, when shorter sampling times were used (Fig. 3a&b). Data obtained by short-time (6 sec) collection were in principal similar to the 1-min sampling experiments (Fig. 3a-c).

Fig. 4 illustrates results obtained from direct counting with the MAS-100 unit on a bacterial lawn in M17 soft agar. At high phage counts in the bioaerosols, total lysis of indicator cells could be seen (Fig. 4a). Figs 4b and 4c show plates still uncountable with almost confluent lysis (Fig. 4b) or plaques still overlapping (Fig. 4c). Only plates represented by Fig. 4d were suitable for direct counting of phage-derived plaques without subsequent serial dilutions. However, all plates instantly allowed the detection of phage present in the indoor air.

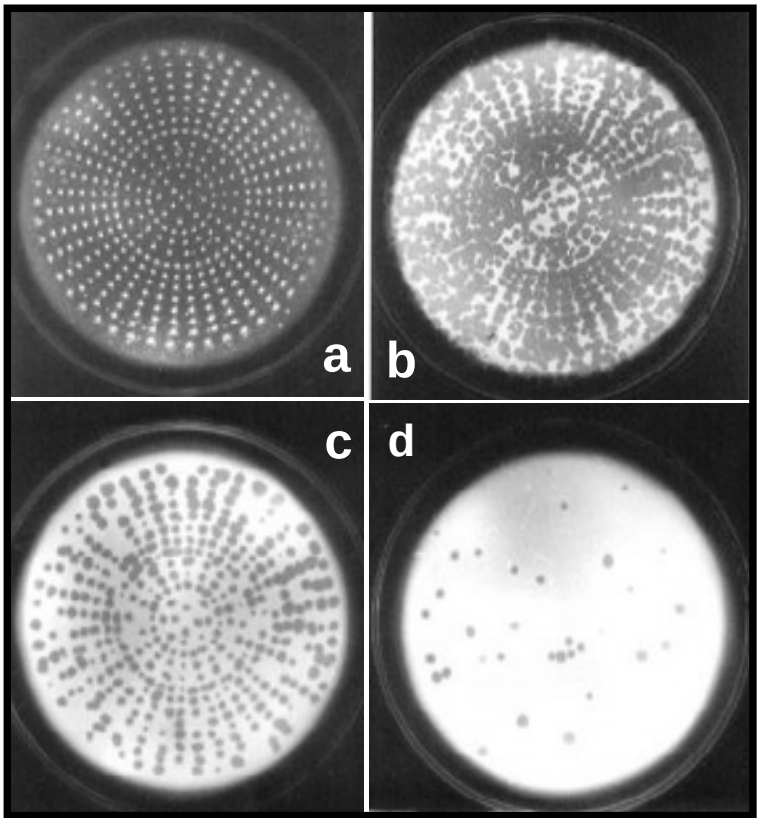

Fig. 4: Illustration of cell lysis in the lawn of a phage-sensitive *L. lactis* indicator strain by phages collected with the MAS-100 unit from air at locations with different phage levels (a to d) (for details, see text). Phages were impacted on M17 soft agar (protocol B3) seeded with indicator bacteria before sampling.

The collecting head of the MAS-100 sampler can be tilted individually from horizontal to vertical positions. Using head tilts of 0° (horizontal position), 45° and 90° (vertical position), parallel samples were taken according to procedure B3 (Table 1, i.e., direct sampling on bacterial lawn in M17 soft agar). The data confirmed that the actual head position did not influence the phage recovery rates (Fig. 5).

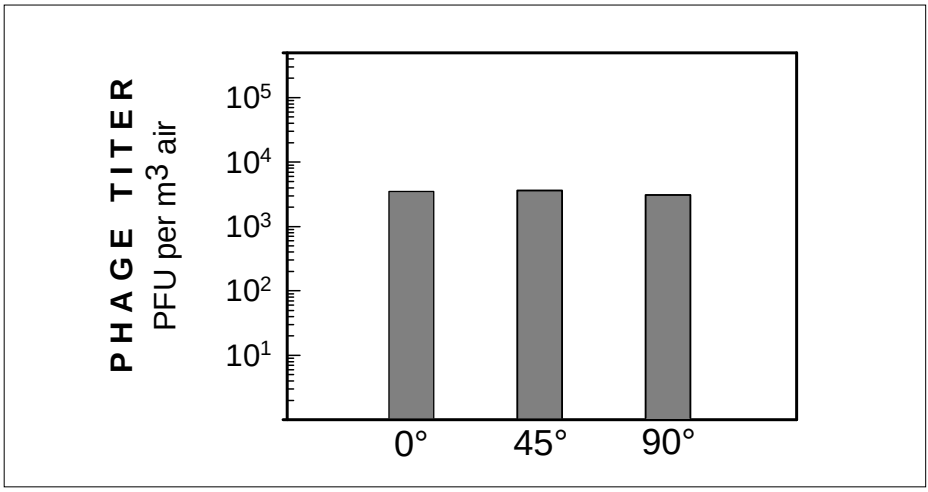

Fig. 5: Recovery of *L. lactis* phages from indoor aerosols by the MAS-100 apparatus at 3 different tilted positions of the collection head.

Next, we tested the applicability of the MD8-specific gelatine filters (14) for collecting airborne phages with the MAS-100 sampler (protocol B5, Table 1). The dry membrane filters were placed in petri dishes. After sampling, the gelatine filters were handled as described for the MD8 and Air Port MD8 units. It is shown in Fig. 6 that this simple technique is also suitable for direct impaction of phages, when compared to the MD8 sampling reference (A) and to the MAS-100 sampling data obtained according to protocols B1 and B2, respectively (Table 1). Again it was noted that the recovery rates obtained by impaction were lower than those obtainedcollected by filtration.

Starter activity test

Phage collected with the MAS-100 unit on gelatine overlay (protocol B2, Table 1) were used for testing the applicability of a starter activity assay. The undefined mixed-strain starter culture, a phage-resistant single-colony isolate, and a phage-sensitive indicator strain were grown in skim milk in the presence of aliquots of the gelatine overlay. For the controls, the gelatine overlay was heated for 20 min at 90°C. Fig. 7 illustrates that acidification of milk by the phage-sensitive indicator strain stopped after 1.5-h incubation when pH 6.1 was reached, although the phage count collected with the MAS-100 device was low (final concentration in the test tube: $9x10^1$ PFU/ml). The original starter culture was not affected by the phage-containing gelatine overlay even at higher phage numbers (final concentration in the test tube: $9x10^4$ PFU/ml). At this phage titer, a minor delay of activity was noted for the phage-resistant derivative after a 2-h incubation. These experiments show that the phage population recovered by impaction with the MAS-100

unit could be used directly for testing starter activity of phage-sensitive *L. lactis* strains. Hence, phage-induced delay in lactose fermentation can be readily scored after a short incubation time of 1.5-2 h, when suitable phage indicator strains are available.

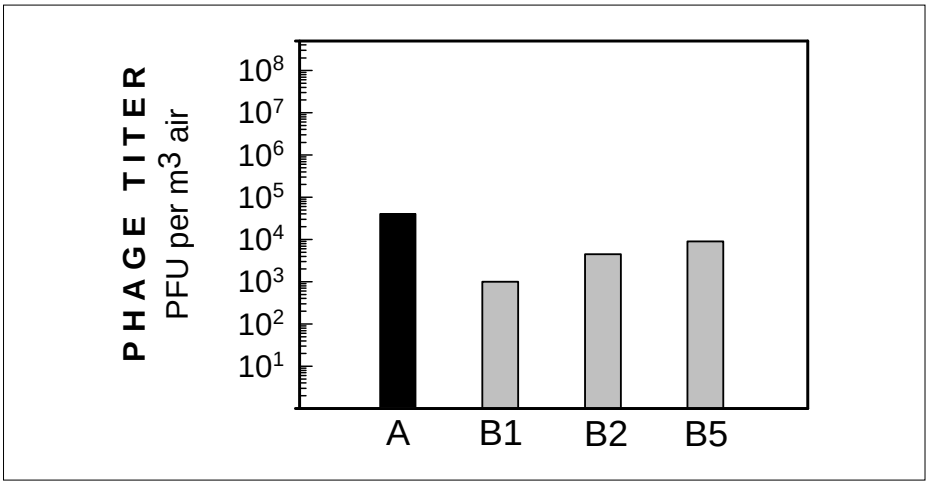

Fig. 6: Comparison of the retrival of airborne *L. lactis* phages with the MD8 unit (A) and the MAS-100 impactor. Three different experimental set-ups were tested for the MAS-100 device (B1, B2, and B5) as indicated in the text and in Table 1. 100-l samples were taken in all cases within 1 min. The MD8-specific gelatine membrane filters were also used in B5 with the MAS-100 unit.

4. Conclusion

Phage-containing bioaerosols can either be dry or wet. Within a dairy plant, the latter type is represented by phage-containing whey. Lactococcal phages generally reveal high stability both in the dry and in the liquid state, respectively (31). We have shown earlier that whey-derived bioaerosols can contain high levels of *L. lactis* phages (up to $2x10^8$ PFU per m^3 air) (14). Hence, whey separators must be regarded as highly effective indoor phage nebulizers. A number of factors have been assessed as being important for the aerosol stability of phages, including relative humidity, temperature, air ions, chemical components of the aerosolised substrate and sample prehumidification (32-37).

A wide range of bioaerosol samplers have been tested in dairy environments (38-40) which are based on liquid impingement, impaction or filtration (28-30). Early studies on *Escherichia coli* phages and other viruses of Gram-negative bacteria in bioaerosols were mainly performed with liquid impingers (28-30). An impaction-based sampler was used for detecting coliphages in bioaerosols (41). In this report, coliphages were concentrated from air within and around poultry broiler houses but their numbers was not determined quantitatively. In our investigation, we demonstrate that the MAS-100 impactor can be used for collecting *L. lactis* phages from indoor dairy air, but the recovery efficiencies are lower as compared with the MD8 sampler. Hence, the latter is suitable for obtaining quantitative data on the dissemination of dairy phages from indoor bioaerosols, while the MAS-100 impactor gives quantitative or semi-qualitative results. The MD8 and the AirPort MD8 samplers are both equally well suitable for counting air-

borne *L. lactis* phages. We also show that *L. lactis* phages recovered from indoor dairy air can be used for a starter activity test. This, however, requires the availability of suitable phage sensitive indicator strains.

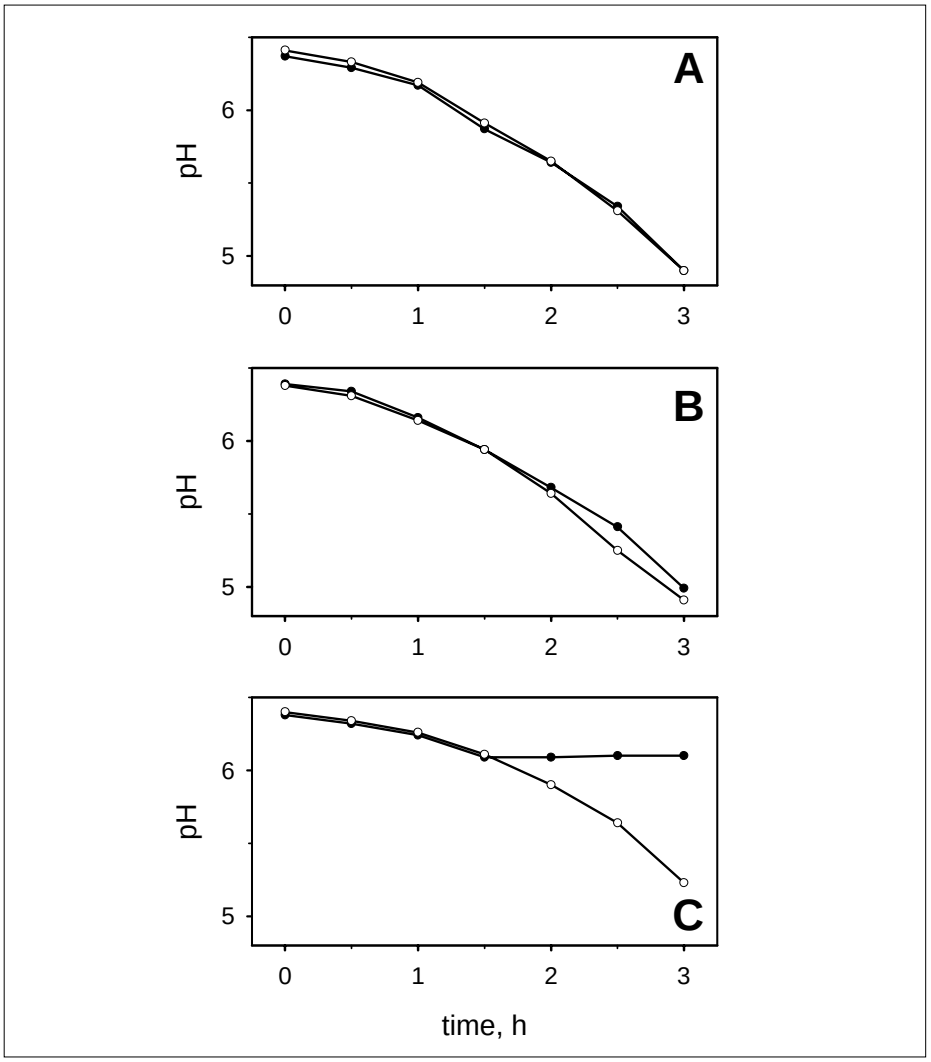

Fig. 7: Acid-producing activity of the undefined mixed-strain starter culture (A), of a phage-insensitive single-colony isolate (B) and of a phage-sensitive isolate (C) in skim milk in the presence of airborne *L. lactis* phages collected with the MAS-100 unit on gelatine overlay. Two phage titers were used for the activity test (final phage titers: A & B: $9x10^4$ PFU/ml; C: $9x10^1$ PFU/ml). Phages were used as retrived (-●-) according to the experimental set-up B2 [see Table 1 and details in text) or were heat-inactivated at 90°C for 20 min as controls (-o-).

In summary, detection and enumeration of dairy phages in bioaerosols is an important aspect of phage monitoring and phage control. Airborne phages have also been investigated intensively by aerobiologist as tracers and indicators e.g. during waste water handling (42-44) and in the interesting research field of phage therapy (45-46).

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6. Summary

Neve, H., Laborius, A., Heller, K.J.: **Testing of the applicability of battery-powered portable microbial air samplers for detection and enumeration of air-borne dairy *Lactococcus lactis* bacteriophages**. Kieler Milchwirtschaftliche Forschungsberichte **55** (4) 301-315 (2003)

26 Microbiology (*Lactococcus lactis* bacteriophages, whey aerosol, air sampling)

Two battery-operated air samplers (AirPort MD8, MAS-100) were used for collecting aerosols in a dairy factory producing fresh cheese, and were compared for their recovery rates of indoor airborne *Lactococcus lactis* bacteriophages. As a reference, air samples were also collected on gelatine filters using an MD8 unit as described earlier (14). A phage-sensitive strain isolated from the undefined mixed-strain starter culture was used as indicator strain for the determination of the phage titers (plaque-forming units [PFU]) per m^3 indoor air). Recovery rates of phages by the portable AirPort MD8 unit on gelatine filters were nearly identical to those obtained with the MD8 reference device. The highest phage numbers were measured close to the whey separator: 5.2×10^6 PFU/m^3 air [MD8] *versus* 4.4×10^6 PFU/m^3 [Airport MD8]): With the MAS-100 impactor, sampling efficiencies were lower (1%-5%) as compared with the MD8 reference unit. The highest

phage numbers measured were $5.4x10^5$ PFU/m^3 air (MAS-100 unit) *versus* $5.2x10^7$ PFU/m^3 (MD8 unit). Phage recovered from bioaerosols by the MAS-100 apparatus were also used for establishing an activity test based on a phage-sensitive starter isolate.

Zusammenfassung

Neve, H., Laborius, A., Heller, K.J.: **Eignungsprüfung batteriebetriebener tragbarer Luftkeimsammelgeräte zum Nachweis und zur Zählung von *Lactococcus lactis* Bakteriophagen in der Raumluft milchverarbeitender Betriebe**. Kieler Milchwirtschaftliche Forschungsberichte **55** (4) 301-315 (2003)

26 Mikrobiologie (*Lactococcus lactis* Bakteriophagen, Molkenaerosol, Luftkeimsammlung)

In einem milchverarbeitenden, Frischkäse-produzierenden Betrieb wurden mit zwei batteriebetriebenen Luftkeimsammelgeräten (AirPort MD8, MAS-100) Aerosolproben gezogen. Die beiden Geräte wurden hinsichtlich ihres Rückhaltevermögens für *Lactococcus lactis* Bakteriophagen aus der Raumluft verglichen. Als Referenz wurden Luftproben mit einem MD8-Gerät nach einer früher erarbeiteten Methode genommen, bei der die Probennahme auf Gelatinefilter erfolgt (14). Zur Bestimmung der Phagentiter (Plaque-bildende Einheiten [PbE]) pro m^3 Betriebsraumluft) wurde ein phagensensitiver Stamm aus der undefinierten Vielstammkultur als Indikatorstamm verwendet. Die mit dem tragbaren AirPort MD8-Gerät ermittelten Rückhalteraten waren nahezu identisch mit denen des MD8 Referenzgerätes. Die höchsten Phagenwerte wurden in unmittelbarer Nähe zum Molkenseparator bestimmt: $5,2x10^6$ PbE/m^3 [MD8] *versus* $4,4x10^6$ PbE/m^3 [Airport MD8]). Die Sammlungseffizienz des MAS-100 Impaktionsgerätes war im Vergleich zum MD8-Referenzgerät niedriger (1%-5%). Die höchsten gemessenen Phagenwerte lagen bei $5,4x10^5$ PbE/m^3 (MAS-100) *versus* $5,2x10^7$ PbE/m^3 (MD8). Weiterhin wurde ein Aktivitätstest mit den Phagen gezeigt, die mit dem MAS-100 Gerät aus Bioaerosolen gesammelt worden waren. Der Test erfolgte mit dem phagensensitiven Starterkulturisolat.

Résumé

Neve, H., Laborius, A., Heller, K.J.: **Examen d'aptitude d'échantillonneurs de l'air portables, opérés à piles, pour la détection et le dénombrement de bactériophages *Lactococcus lactis* dans l'air ambiant des entreprises de transformation de lait**. Kieler Milchwirtschaftliche Forschungsberichte **55** (3) 301-315 (2003).

26 Microbiologie (*Lactococcus lactis* bactériophages, lactosérum en aérosol, échantillonnage de l'air)

Dans une entreprise de transformation de lait (production de fromage frais), des échantillons en aérosols ont été pris avec deux échantillonneurs d'air opérés à piles (AirPort MD8, MAS-100). Les deux appareils ont été comparés sur leur capacité de recouvrement de bactériophages *Lactococcus lactis* de l'air ambiant. Les échantillons d'air étaient pris avec un appareil MD8 selon une méthode antérieurement élaborée où l'échantillonnage se fait sur filtre en gélatine (14). Pour déterminer le titre de phage

(unités formant plaques [UFP]) par mètre cube d'air ambiant de l'entreprise, une souche à phage sensible de la culture indéfinie à levains mélangés a été utilisée comme souche indicatrice. Les taux de recouvrement mesurés avec l'appareil portable AirPort MD8 étaient quasi identiques avec ceux obtenus avec l'appareil de référence MD8. Les valeurs de phages les plus élevées ont été détectées dans l'environnement direct du séparateur de lactosérum : $5{,}2 \times 10^6$ UFP/mètre cube [MD8] *contre* $4{,}4 \times 10^6$ UFP/mètre cube [Airport MD8]). L'efficacité d'échantillonnage de l'impacteur MAS-100 était moins élevée que celle de l'appareil de référence MD8 (1%-5%). Les valeurs de phages les plus élevées étaient $5{,}4 \times 10^5$ UFP/mètre cube (MAS-100) *contre* $5{,}2 \times 10^7$ UFP/mètre cube (MD8). Des phages recueillis d'aérosols biologiques avec l'appareil MAS-100 ont également été utilisés pour établir un test d'activité sur un isolat de levain à phage sensible.