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Survival of African swine fever virus in feed, bedding materials and mechanical vectors and their potential role in virus transmission

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Abstract

Over the last years, African swine fever (ASF) has gone pandemic and within the European Union affected wild boar populations are main drivers. This brings new challenges, i.e. risk assessment needs for agricultural products and the role of mechanical arthropod vectors. Answering the call "Survival of African swine fever virus in feed, bedding materials and mechanical vectors and their potential role in virus transmission" (GP/EFSA/ALPHA/2021/09), relevant feed and bedding materials were chosen for stability experiments. All matrices were contaminated with ASFV and stored at five different ambient conditions (-20°C, 4°C, 10°C, 20°C, and 37°C) over a period of up to nine months. Replicate samples were evaluated at different time-points using real-time PCRs and virus isolation. Additionally, the possible role of three types of blood-sucking arthropods was assessed. In detail, studies were carried out on how long representative arthropods harbored viral genome and infectious virus upon feeding on infectious blood. In a last step, further proof-of-concept data were generated on the transmission of ASFV via ingestion of (small) arthropods after an infected blood meal. Concluding, detection of infectious virus was rather limited in most matrices while detection of viral genome was possible over the entire study period. At lower temperatures, however, the virus was stable on feed matrices over several days or even weeks, especially on beet and potatoes. Grass, grass silage and corn silage did not allow re-isolation of virus at any time-point. The studies on the detectability of the virus in arthropods showed that the virus is generally detectable for a certain period of time depending on temperature and ingested volume. The detectability of virus in stable flies exceeded the expectations with over 168 hours at cool temperatures. The feeding experiment did not lead to infection of pigs. However, the power of this proof-of-concept study is limited.

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Keywords: African swine fever virus, stability, feed, bedding, arthropods, transmission

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Summary

Over the last years, African swine fever (ASF) has gone pandemic and within the European Union affected wild boar populations are main drivers of the epidemic. This situation brings new challenges, i.e. risk assessment needs for agricultural products harvested in affected regions with a risk of being exposed to carcasses or body fluids of affected animals. Answering the call "Survival of African swine fever virus in feed, bedding materials and mechanical vectors and their potential role in virus transmission" (GP/EFSA/ALPHA/2021/09), the consortium set out to provide reliable data on the stability and inactivation (survival) of ASF virus (ASFV) in plant derived feed and bedding materials. Considering production parameters in Europe, 14 relevant feed and bedding materials, i.e. grass, grass silage, hay, barks, peat, wood shavings, corn silage, rapeseed, barley, wheat, oats, straw, potatoes and fodder beet were chosen for detailed stability experiments. All matrices were manually contaminated with ASFV and stored at five different ambient conditions (-20°C, 4°C, 10°C, 18-22°C, 37°C) over a period of up to nine months. Replicate samples (biological triplicates tested in at least three technical runs) were evaluated at different time-points using real-time PCRs for viral genome detection and virus isolation on susceptible primary and/or permanent cells. To ease evaluation of virus isolation, a cell-culture adapted and/or fluorescence labelled ASFV strain spiked into negative pig blood was used for screening purposes. Critical time-points were repeated with a most recent German ASFV strain (blood from an infected animal). The studies were rounded off with proof-of-concept studies targeting risk mitigation through the use of organic acids on hay, straw, and wheat.

The study showed that methodology is a critical issue when conducting stability testing. Established systems using primary cells provided the most sensitive results, despite technical hurdles, and are therefore preferable.

The stability of the viral genome was expected to be very high, and it is therefore not surprising that the viral genome could be detected over a wide temperature range and long periods of time. The detection of genomes from silage, grass and hay was difficult. These problems could only be partially overcome by adapted extraction methods. We assume that these inhibitory effects still indicate very rapid inactivation.

The situation is different for the detection of the infectious virus. Despite several attempts, the infectious virus could only be detected in a few samples and only at cool temperatures. Surprisingly, often the virus was detected in/on beet and potatoes. Here, the virus was recovered for several weeks at cool temperatures that would be representative for late autumn.

Similar results were obtained with bedding material. Detection of the virus was limited to cool storage temperatures and short periods of time. At 4°C, inactivation usually occurred after 7 days, with the exception of one bark sample that remained weakly positive after 28 days. Even at 10°C, only the bark allowed a single detection after 7 days. Storage at moderate temperatures was always suitable for inactivating the virus under the experimental conditions. Treatment with propionic acid had little additional mitigation effect, which correlates with the generally low detectability.

While it is acknowledged that only soft ticks of the genus *Ornithodoros* can act as competent biological vectors for African swine fever virus (ASFV), i.e. replicate the virus and maintain it over their stages, the possible role of other blood-sucking arthropods as mechanical vectors is still controversially discussed. In particular, the seasonality observed in the current ASF

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epidemic with a very clear trend of outbreaks in domestic pig farms occurring during summer months, raised the question on whether arthropod vectors could play a role in disease dynamics, as a bridge between an infected wild boar population and domestic pigs or within and across domestic pig farms. The research consortium aimed at providing reliable data on role of mechanical vectors in ASFV transmission. In detail, studies were carried out on how long representative arthropods will harbor infectious virus upon feeding on infectious blood. To this means, laboratory colonies of *Aedes albopictus* and *Stomoxys calcitrans* were investigated along with tabanids caught in the field.

The studies showed that the virus is generally detectable for a certain period of time depending on temperature and ingested blood volume. As expected, the virus is detectable in Stomoxys flies for a relatively long time, at 10°C even significantly longer than expected, i.e. over 168 hours. At cool temperatures, the infectious virus could also be detected in mosquitoes for up to 120 hours, which is also longer than anticipated and longer than the expected time of complete blood meal digestion.

To provide additional data on the biological relevance of arthropod ingestion, especially with small arthropods, six domestic fattening pigs were fed with 16 ASFV contaminated Aedes mosquitoes each. The pigs were monitored for fever and ASF related clinical signs for a period of 21 days. At seven days post feeding, a blood sample was taken to investigate for viral genome and various organs and blood were tested upon necropsy. In addition, serology was performed on serum samples taken at day 21. None of the pigs showed viral genome in blood and organs or seroconverted. However, the power of this proof-of-concept study is limited due to the small sample size.

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1 Introduction

Over the last years, African swine fever (ASF) has gone pandemic [1] and within the European Union, affected wild boar populations are main drivers of the epidemic [2]. This situation poses new challenges, i.e. the need for risk assessment of agricultural products harvested in affected regions where there is a risk of exposure to carcasses or body fluids of affected animals. The risk may arise from direct contact with susceptible pigs (feeding or use as enrichment material) or from the movement of contaminated matrices into free regions with potential contact with domestic or wild suids. In the case of contact with susceptible suids, infection biology must be considered. Despite the fact that the oral or oro-nasal route of transmission is much less effective than the parenteral route [3], studies have provided evidence that low amounts of virus can be sufficient to infect susceptible pigs, especially if the exposure is repeated [4, 5]. Thus, any potential contamination with infectious virus can be critical, especially when considering the rather high tenacity of ASFV [6].

With this being said, reliable and applicable data on the survival of the ASF virus (ASFV) in different matrices, on the effectiveness of inactivation procedures and on disinfection possibilities are required for advising risk managers.

Another question that is discussed very frequently is the role of arthropods for the transmission of ASFV. While it is acknowledged that only soft ticks of the genus *Ornithodoros* can act as competent biological vectors for ASFV, i.e. replicate the virus and maintain it over their stages and across generations, the possible role of other blood-sucking arthropods as mechanical vectors are still controversially discussed [7]. In brief, the seasonality observed in the current ASF epidemic with a very clear trend of outbreaks in domestic pig farms occurring during summer months, raised the question on whether arthropod vectors could play a role in disease dynamics, as a bridge between an infected wild boar population and domestic pigs or within and across domestic pig farms.

So far, there is no evidence that blood-sucking arthropods play a significant role in the disease dynamics [7, 8], but in affected farms, trace amounts of viral DNA could be found in arthropods [9] and experimental evidence exists that *Stomoxys calcitrans* could transmit the disease via their contaminated mouth pieces [10] or when ingested [11]. Fila et al. (2020) also discussed the housefly (Musca domestica). For this reason, applicable data are needed to estimate the risk of this potential transmission pathway.

1.1 Background and terms of reference as provided by the requestor

The contract entitled "Survival of African swine fever virus in feed, bedding materials and mechanical vectors and their potential role in virus transmission" (grant number: GP/EFSA/ALPHA/2021/09) was awarded to the Consortium coordinated by the Friedrich-Loeffler-Institute (FLI), Germany. Partners were The German Federal Research Institute for Risk Assessment (BfR), and the Statens veterinärmedicinska anstalt (SVA), Sweden.

The project covered the main objectives of the call, i.e. to assess the **duration of ASFV survival in plant materials** (objective 1) and to assess the **role of mechanical vectors in ASFV transmission** (objective 2).

Answering the call's objective 1, two tasks were set:

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- **Task 1.1 Stability of ASFV in relevant feed and bedding materials**

Production parameters such as cultivated areas in North and Central Europe, average yields per hectare and harvest volumes were taken into consideration when choosing representative matrices. In addition, matrices were selected that were either discussed in the literature and epidemiological studies (e.g. grass [12]) or were the subject of expert opinions requested from different national reference laboratories within the EU (e.g. silage and cereals).

Based on these assessments, stability of ASFV in the following plant-derived feed and bedding materials was assessed: Grass, rapeseed (relevant crop in affected regions, especially in central Europe, high share in protein sources), corn and grass silage (relevant in ASF affected regions, repeatedly subject to requests), potatoes and fodder beet (relevant crop, no existing data), wheat (relevant unhulled grain after threshing, pre-existing data as a starting point), barley (particularly relevant grain in Sweden and other Scandinavian countries), oats (relevant hulled grain, no published data), hay and straw (relevant roughage, discussed as source of infection [7]), barks, peat and wood shavings.

- **Task 1.2 Pilot studies on risk mitigation concepts**

African swine fever in wild boar is currently spreading steadily and affecting ever larger agricultural areas, the yield of which cannot simply be harmlessly removed or excluded from feeding to susceptible animals. Against this background, virus inactivation procedures that guarantee risk minimization must be tested. After discussion with stakeholders of the feed production chain, the use of organic acids, i.e. propionic acid was chosen for a proof of concept trial.

The following priorities and aims were chosen to answer the call's objective 2:

- **Task 2.1 Investigate how long ASFV can be detected in an infected insect blood meal**

Within this task, the question was investigated how long representative arthropods can harbor infectious virus upon feeding on infectious blood. To this means, laboratory colonies of *Aedes albopictus* and *Stomoxys calcitrans* were investigated along with tabanids caught in the field.

To design meaningful studies, the following considerations were included (Figure 1): After completion of a full blood meal, hematophagous arthropods seek a suitable resting site to digest their blood meal. In this context, oral intake of an infectious arthropod by a wild boar is possible, mechanical transmission unlikely. In mosquitoes, it takes 3 to 5 days until the blood is digested and a new host is searched for. However, if the feeding mosquito is disturbed and feeding is interrupted, new hosts are searched for and both oral ingestion and mechanical transmission are possible. In stable flies, digestion is usually completed in several hours and the flies will feed at least once a day or even several times a day during warm conditions. In this case, both oral intake and mechanical transmission are possible. In horse flies, biting is often interrupted due to the painful bite and the host's defensive responses. Therefore, both oral intake and mechanical transmission are possible. In general, mechanical transmission is dependent on multiple factors linked to the biting behavior of the arthropod, their anatomical characteristics, and the climatic conditions.

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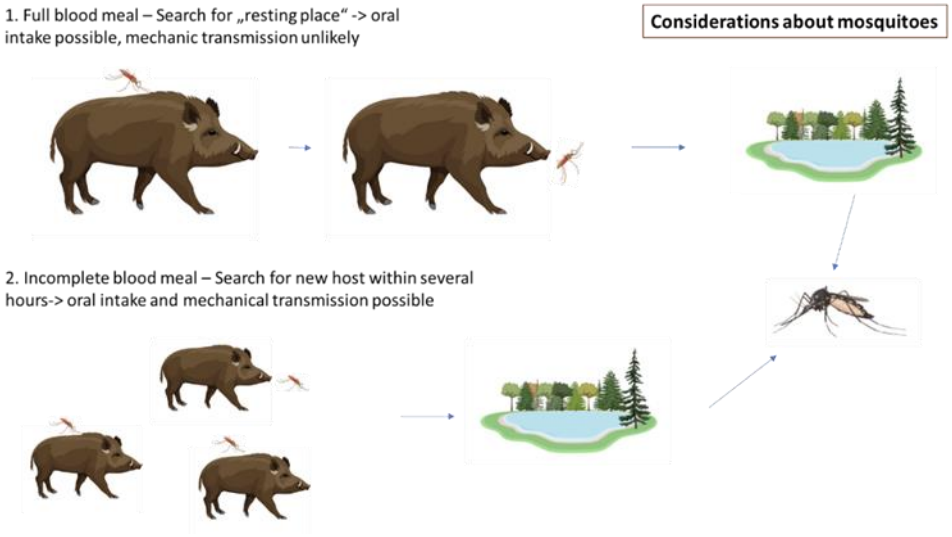

Figure 1: Considerations about mosquitoes as mechanical vectors in the wild boar setting

Three insects with different body and, accordingly, blood meal sizes, were selected to evaluate the role of mechanical vectors. These are the mosquito (*Culicidae*), a small insect, the stable fly (*Muscidae*), a medium-sized insect, and the horse fly (*Tabanidae*), a large insect.

- **Task 2.2 Pilot study on the transmission of ASFV via ingestion of arthropods after an infected blood meal**

In a worst-case scenario, feeding of 6 pigs with 16 small arthropods, i.e. Aedes mosquitoes, each, was performed after an ASFV contaminated blood meal.

2 Methodologies

2.1 ASFV survival in plant materials

The work related to survival in plant materials was carried out under Work Package 1, Stability of ASFV in relevant feed and bedding materials. While the overall stability was assessed under task 1.1, pilot studies on mitigations concepts were carried out under task 1.2.

The ASFV stability on feed (plant origin) and bedding material was tested with two different ASFV-strains, a fluorescent ASFV (ASFV1) to ease reading of results, and a genotype II ASFV from a German case in 2020 (ASFV2). Repeated trials were done with an additional cell-culture adapted ASFV (derived from the genotype II strain “Armenia 2008”). In detail, blood from a negative donor spiked at a ratio of 1:5 with a cell culture adapted ASFV strain with a fluorescent marker (“ASFVΔ258L_GFPhuCD4”) or blood from experimentally infected animals

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(ASFV genotype II strains isolated in Saxony and Brandenburg; "ASFV Germany") was used to contaminate the respective matrices (see below).

Sampling times and study duration mirrored previous, published studies on similar subjects and considered common practices in the field and secondary factors such as the shelf life for perishable feed matrices such as grass. Three biological replicates per temperature and time-point were analyzed. The cell culture adapted; fluorescently labelled virus was used to screen the whole set of samples on a well-defined cell culture system. Due to stability and matrix problems of the fluorescently labelled virus on several matrices, retrials with a tissue culture adapted ASFV strain (ASFV Armenia WSL adapted P23) were performed for wheat, saw dust, hay, bark, peat and grass silage. Furthermore, two representative time-points (7 and 28 days) were repeated using the field strain contaminated materials where the analyses have to be run on primary porcine macrophages (field strains are not adapted to cell culture). No indications exist that the general physicochemical characteristics differ between field and laboratory strains of ASFV. However, the fluorescence was influence by matrix properties, and the respective stock titres were lower. Where process parameters are important, i.e. silage production, ASFV-free blood was used as control.

Temperatures and time-points were in line with the call, i.e. -20 °C, 4 °C, 10 °C, room temperature (18-22 °C), and 37 °C. However, the following deviations were implemented: As stability at -20 °C can be expected for all matrices, only end points, i.e. nine or six-month values were considered, respectively. Given previous experience, stability at 37°C was reduced to early and representative time-points for all matrices. Low temperatures were left out if season of harvest and production parameters suggested that these matrices would not be used under those conditions. Stability on grass was only followed over a two-week period due to the short shelf life. In this way, all critical time-points were considered, and meaningful replicates were possible with the short time frame and budget constraints.

Prior to the below mentioned studies, workflows were adjusted and optimized using different volumes of the matrix and the contaminant, different ways of contamination (spraying, dropping), storage options (bag, box, tube etc.) and pre-treatments. Filtration and reduction of organic compounds with commercial columns was tried. The later was discontinued due to loss in titer and only small beneficial effects on cytotoxicity. An example for such an optimization process is provided in Figure 2.

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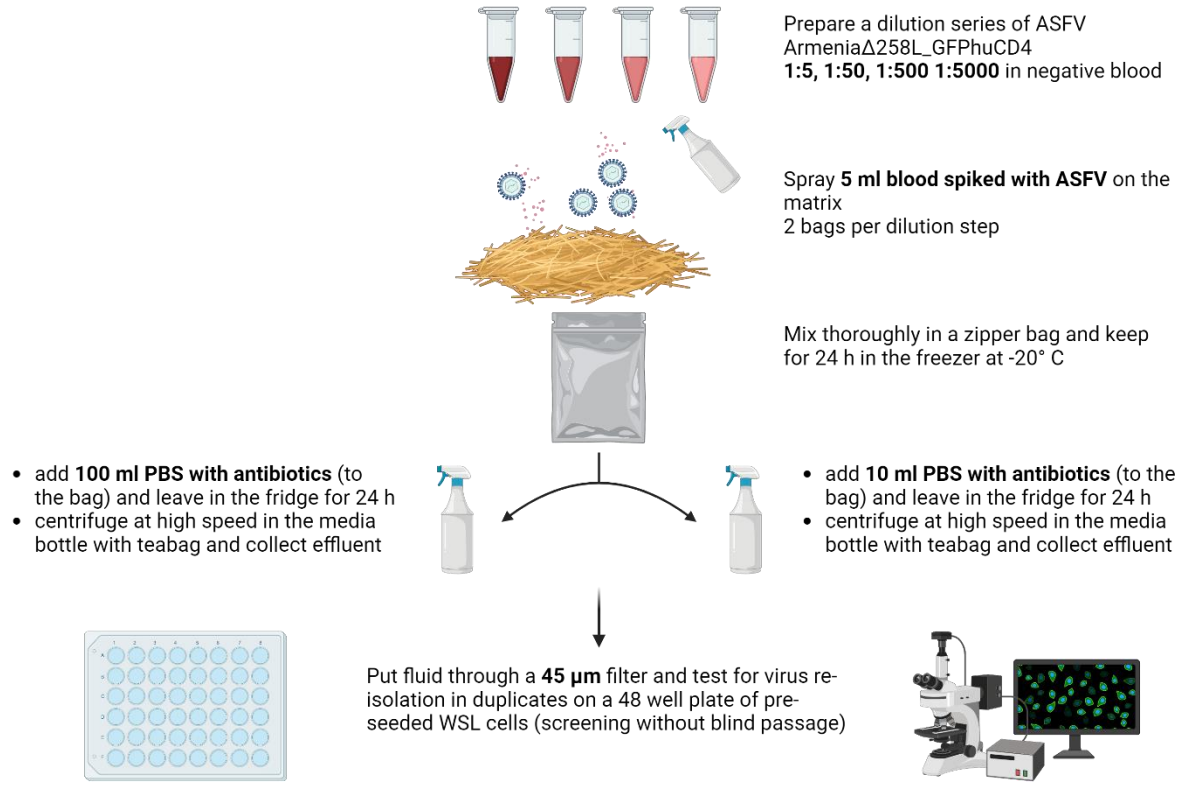

Figure 2: Example for proof-of-concept trials to establish the most sensitive workflow. Compromises have to be found avoiding high toxicity for cell cultures while preserving sensitivity. The visualization was created with BioRender.com.

2.1.1 Grass silage and corn silage

A second cut mixed grass sward (consisting mainly of *Lolium perenne, Dactylis glomerata, Alopecurus pratensis, Arrhenatherum elatius* and *Poa annua*) was harvested at booting stage in the morning of June 8th 2022 in Brandenburg, Germany. It was cut to approximately 4 cm chop length particles and directly transported to FLI in Riems, Mecklenburg-Western Pomerania, Germany, for silage preparation. Maize/corn (*Zea mays*) was harvested in the morning of the 7th of October 2022 at a field near Riems, chopped to particle length of 2 cm and also transported to FLI. Grass and corn silages were prepared in laboratory scale silos by using 1,5 l Weck jars and stored over 3 to 180 days at -20°C, 4°C, 10°C, 20°C or 37°C. Silage preparation was described previously and followed standard guidance (DLG, 2020; Klevenhusen et al., 2022 [13]).

In detail, for individual silage preparation, approximately 1 kg of either grass (GS) or maize (MS) were inoculated and thoroughly mixed with different additives in open transparent plastic boxes. Treatments were: (1) CON, control treatment with homofermentative lactobacilli at 3 x 10^5 cfu/kg plus 30 g molasses/kg and 10 ml ASFV-free blood; (2) ASFV1, 10 ml homofermentative lactobacilli at 3 x 10^5 cfu/kg plus 30 g molasses/kg and 10 ml of fluorescent ASFV blood; (3) ASFV2, 10 ml homofermentative lactobacilli at 3 x 10^5 cfu/kg plus

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30 g molasses/kg and 10 ml of ASFV "Germany 2020" blood. The silage mixture was then filled into the empty weighed jars, compacted as well as sealed airtight and final weight recorded. Samples were prepared as triplicates for each treatment, storage temperature and storage time. An overview of storage conditions and sampling scheme for the silages is shown in Table 1.

Table 1: Scheme of storage duration and temperature of the grass and maize silages (CON)

Temperature	Ensiling time
Grass/Maize silage	
- 20°C	180 days
4°C[a]	3, 7, 14, 28, 60, 90 and 180 days
10°C	3, 7, 14, 28, 60, 90[b] and 180[c] days
20°C	3, 7, 14, 28, 60 and 90 days
37°C	3, 7, 14 and 28 days

[a] for maize silage only; [b] 2 jars for maize silage; [c] 1 jar for maize silage

After a pre-defined storage time (Table 1), all three jars per CON treatment were slightly opened and weighed for determination of dry matter (DM) loss according to Weissbach and Strubelt, 2008. Subsequently the jars were opened, homogenized and samples taken for analysis of nutrients, pH, ammonia-N (NH_3-N), fermentation acids and alcohols. Visibly deteriorated silage was excluded from analysis. Samples were stored at -20°C until analysis. An example of the storage is depicted in Figure 3.

Figure 3: Example for silage processing. Preserving jars were used for the ensiling process at different ambient temperatures. To assess the impact of the blood matrix on the process, negative samples were tested along with truly contaminated ones.

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Under containment conditions, jars of grass silage were opened at sampling days and the first 2-5 cm of the silage were discarded. The remainders were transferred to zipper bad and frozen for >24 h at -20°C to optimize the release of the contaminants.

The pH of the grass silage was recorded for each sampling day. For this purpose, 50 g silage were mixed with 200 ml Aqua bidest., stored over night at 4°C and measured using a laboratory pH meter. To the other part of the silage, 50- 100 ml of phosphate buffered saline were added and thoroughly mixed (depending on the dry matter content). A salad spinner was be used to retrieve washing fluid. To harmonize subsequent testing in qPCR and virus isolation, fluids were stored at –80°C until further use. For the use in cell culture, fluids were filtrated through a 45 µm syringe filter.

Storage temperatures comprised -20°C (only end point), 10°C, room temperature (18-20°C), and 37°C as mentioned above. The storage at 4°C was left out due to harvest time and ambient temperature in the critical phase of the ensiling process (in summer). Samples for the overall screening were harvested at days 0, 3, 7, 14, and 28 for all temperatures, at days 60 and 90 for 10°C and room temperature, and at 180 days for samples stored at 10°C. Days 7 and 28 were repeated with field virus contaminated silage.

Furthermore, additional tests were performed due to stability problems of the fluorescent virus on silage. To this end, grass silage (28 dpi 10°C) produced at BfR was heavily contaminated with blood spiked with cell culture adapted ASFV Armenia (2 ml per sample). Thereafter, 4 g aliquots were filled into 50 ml falcon tubes and stored at 10°C. After 0, 1, 2, 3, 4, 6 and 8 hours, the aliquots were stored at -80°C until further processing. For downstream analyses, the silage was transferred to a falcon tube equipped with three 0.5 ml Eppendorf tubes to allow fluid to flow through to the bottom. After adding 10 ml of PBS, the silage-fluid mixture was vortexed and left at 4°C for 30 min. After ultrasound treatment for 5 min, the samples were again vortexed and centrifuged at 3023 rcf for 15 in at 4°C. The fluid collected from the bottom of the tube was aliquoted for PCR (kept at -20°C) and virus isolation (stored at -80°C).

For the corn silage, a similar protocol was used. In detail, the pH of the silage was recorded for each sampling day. For this purpose, 50 g silage was mixed with 200 ml Aqua bidest., stored over night at 4°C and measured using a laboratory pH meter. The remainder was stored at -20 °C for >24h, mixed with 100 ml of phosphate buffered saline and incubated for 10 min at room temperature. To collect the liquid, the mixture was centrifuged for 15 min at 3023 rcf at 4°C. A salad spinner was used to retrieve additional washing fluid. Subsequently, silage was placed in cotton teabags and centrifuged for 15 min at 3023 rcf at 4°C. To harmonize subsequent testing in qPCR and virus isolation, fluids were be stored at –80°C until further use. For the use in cell culture, fluids were filtrated through a 45 µm syringe filter.

2.1.2 Grass, potatoes, fodder beet, hay, and bedding materials

In addition to grass silage and maize silage, investigation on the viability of ASFV was performed on fresh grass (botanical composition and chop length as described above), on fresh potatoes, fodder beet and hay harvested from the area around Riems as well as commercial barks (pine, JBL Terrabark 10-20 mm), peat (Wühlerde, Moorsol®) and saw dust (DM Kleintierstreu) purchased on the market.

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To this means, portions of 500 g **grass** and 10 ml contaminant were be placed in open transparent plastic boxes to avoid mold growth. At sampling days, the grass was transferred to zipper bags and frozen for >24h at -20°C to optimize release of the contaminant. Thereafter, 50 - 100 ml of phosphate buffered saline were added and thoroughly mixed. A salad spinner was used to retrieve washing fluid. To harmonize subsequent testing, fluids were stored in conical tubes at –80°C until further use. For the use in cell culture, fluids were filtrated through a 45 µm syringe filter. Grass was kept solely at 10°C and room temperature. These temperatures mimic the ambient temperatures at the time of potential harvest and feeding. Samples were taken at days 0, 1, 3, 7 and 14 for the fluorescent virus and at day 7 for the field virus.

Samples of **hay, bark, peat and sawdust** were aliquoted to comprise 40 g and contaminated with 5 ml of the respective virus suspension (blood spiked with fluorescent virus or blood containing ASFV field strain) in a zipper bag. Storage took place in small, open plastic boxes to avoid mold growth (see figure 4). At sampling days, matrices were transferred to freezer bags and stored for >24h at –20°C to optimize release of the contaminant. Thereafter, 200 ml Aqua dest. were added, thoroughly mixed and incubated for 24 h at 10°C. To harvest the washing fluid, the wet matrices were placed in cotton teabags (Fenshine single use tea bags, 10 x 15 cm Sea&Mew) and centrifuged in a 500 ml cell culture flask with pointed bottom. As above, fluids were be stored at –80°C until further use. For the use in cell culture, fluids were filtrated through a 45 µm syringe filter.

Figure 4: Storage of contaminated enrichment and bedding materials. Open boxes ensure reduction of mold growth. To standardize the storage conditions, a stack is closed off by an empty box or a plastic disc (not shown here). All boxes face similar directions.

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All matrices for the overall screening were investigated over the entire six to nine-month study period when stored at 4°C and 10°C. Samples stored at room temperature were followed for 90 days, the 37°C samples over 28 days (see table 2). Field virus were evaluated at days 7 and 28.

Due to stability and matrix problems of the fluorescent virus, a retrial with cell culture adapted ASFV Armenia was performed for hay, bark, peat and sawdust. Therefore, these matrices were contaminated with blood spiked with ASFV Armenia P23. After that 4 g of each matrix were aliquoted into small boxes and stored at the above-mentioned temperatures. At the date of sampling the aliquot was transferred into a 50 ml falcon tube and stored at -20°C until further processing. For release of the contaminant 15 ml of phosphate buffered saline were added to the falcon tubes. Afterwards tubes were thoroughly vortexed, put in an ultrasound bath for 5 min and incubated for 30 min at 4°C. Before collection of the washing fluid, tubes were vortexed again. Aliquots for PCR were stored at -20°C and aliquots for cell culture at -80 °C.

Potatoes for our study were harvested in September. As above mentioned, samples were stored at all ambient temperatures (-20°C only end point). Samples were taken at days 0, 3, 7, 14, and 28 for all storage temperatures, in addition at days 60 and 90 for storage conditions 4°C, 10°C and room temperature, and at days 120, 180 and 274 for 4°C and 10°C (see table 2). Days 7 and 28 were repeated with field virus.

To contaminate the potatoes, they were placed in zipper bags and contaminated with 10 ml of the respective virus suspension (blood spiked with fluorescent virus or blood containing ASFV field strain). Storage was done in open plastic boxes, where possible in the dark. At sampling days, potatoes were placed in a freezer bag, 200 ml Aqua bidest. were added and potatoes were incubated >24h at 10°C. Afterwards potatoes were scrubbed/rubbed with a sponge (our special thanks go to E. La Griega and S.A. Koronis who have established a similar protocol for environmental and animal-based samples) soaked with phosphate buffered saline. The washing fluid was collected by centrifugation for 15 min at 3023 rcf at 4°C. To harmonize subsequent testing in qPCR and virus isolation, fluids were stored at –80°C until further use. For the use in cell culture, fluids were filtrated through a 45 µm syringe filter.

Fodder beet was harvested in October 2022. Due to the storage conditions in the field, only 4°C storage was investigated over the entire period of 180 days. Field virus was added for days 7, 28, 90 and 120.

As above mentioned, beets were placed in zipper bags and contaminated with 10 ml of the respective virus suspension (blood spiked with fluorescent virus or blood containing ASFV field strain). Storage was done in open plastic boxes, where possible in the dark. At sampling days, beets are placed in a freezer bag, 200 ml Aqua bidest. were added and incubated >24h at 10°C. Afterwards beets were scrubbed/rubbed with a sponge soaked with phosphate buffered saline (see above). The washing fluid was collected by centrifugation for 15 min at 3023 rcf at 4°C. To harmonize subsequent testing in qPCR and virus isolation, fluids were stored at –80°C until further use. For the use in cell culture, fluids were filtrated through a 45 µm syringe filter.

Table 2 gives an overview of storage conditions and sampling scheme for the feeds of plant origin, except silages, and the bedding materials.

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Table 2: Scheme of storage duration and sampling for feed (plant origin) and bedding material

Day	Grass, potatoes, fodder beet, hay, barks, peat, saw dust							
	ASFV1				ASFV2			
	4°C	10°C	20°C	37°C	4°C	10°C	20°C	37°C
3	X	X	X	X				
7	X	X	X	X	X	X	X	X
14	X	X	X	X				
28	X	X	X	X	X	X	X	X
60[a]	X	X	X					
90	X	X	X					
120	X	X						
180	X	X						
274[b]	X	X						

[a] final sampling of fodder beets; [b] final sampling of grass, potatoes, hay, barks, peat and saw dust

At SVA, barley, oats, rapeseeds, wheat, and straw were purchased from a local farm in Uppsala, Sweden. Fifteen milliliters of grains were placed in a 50-ml falcon tube and fifteen grams of straw were placed in a zip plastic bag. Pig EDTA-blood was mixed with ASFV strain BA71V to obtain a 10^6 $TICD_{50}$ virus titer. The grains were contaminated with 150 µl of the spiked blood and mix thoroughly by shaking the tubes. The straw was added with 1000 µl of the spiked blood. The matrices were also contaminated with the same amounts of a pig blood sample positive for ASFV provided by FLI. Additional wheat and straw were contaminated the blood samples and 160 µl of propionic acid (ca 1% of the matrices) was added to the wheat or sprayed to the straw to study virus inactivation. Both matrices were mixed well by shaking the tubes or "massaging" plastic bags thoroughly. Each treatment had three replicates. The outer surfaces of the tubes and bags were disinfected with 2% Virkon®. The matrices were stored at given temperature conditions and upon completion, they were stored at -20 or -70°C freezer prior to further process. Part of acid-treated matrices were processed on the sampling days by PD-10 desalting columns (Cytiva, Uppsala, Sweden) followed by 0.45 µm filtration.

Filtration through a 0.45 µm filter was selected as the most appropriate method to elute viruses from the matrices after a thorough comparison. Grans were added with 25 ml of PBS and straw with 50 ml and mixed well. A 0.8 µm filter was used to remove larger substance from straws. The elutes were stored at -20°C.

2.2 Role of mechanical vectors in ASFV transmission

Mosquitoes (Culicidae)

For the *Culicidae* the mosquito species *Aedes albopictus* was chosen. Several colonies of this species are available in the insectaries of the FLI. This species can be raised in large number, it has a wide host range and aggressive biting behaviour and gains importance also as an invasive species expanding northwards. It is for these reasons very suitable as a model species for the experiments in this task.

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Mosquito rearing techniques

Aedes albopictus were uniformly reared under controlled conditions (26 °C ± 2 °C, 70% ± 10% relative humidity, and 16:8 light-dark photoperiod) in the insect collection of the Institute of Infectiology, Friedrich-Loeffler-Institute, Germany. Adults were provided with cotton rolls soaked in 5% glucose solution on the first day after hatching and kept in mosquito-proof cages (30 cm [L] × 30 cm [W] × 30 cm [H]) with a steel mesh on top for blood feeding. Female mosquitoes were allowed to feed on bovine blood via an artificial membrane feeding system (Hemotek). A urine cup containing moist filter paper was placed in the cages for egg deposition. Eggs were dried at room temperature and stored up to 6 months before being immersed in ToruMin water in individual hatching trays. Larvae were fed a commercial ground fish food (TetraMin® XL Flakes) on Mondays, Wednesdays and Fridays. Pupae were transferred from larval trays to hatching cups on Mondays, Wednesdays, and Fridays and placed directly into mosquito-proof cages. Hatching adults were kept in cages with a 5% glucose solution for 10 days prior to the experiments.

Stable fly (Muscidae)

Laboratory colonies of the stable fly *Stomoxys calcitrans* provided by MSD Animal Health Innovation GmbH were chosen, because they have previously been described as mechanical vectors for several pathogens [4].

Stomoxys rearing techniques

Pupae of *Stomoxys calcitrans* were received from MSD Animal Health Innovation GmbH and allowed to hatch in fly-proof cages (30 cm [L] × 30 cm [W] × 30 cm [H]) with a steel mesh on top for blood feeding. The adults were kept under controlled conditions (26 °C ± 2 °C, 70% ± 10% relative humidity, and 16:8 light-dark photoperiod) in the insect collection of the Institute of Infectiology, Friedrich-Loeffler-Institute, Germany. For the first 5 days, the adult Stomoxys were fed a 3% glucose solution. When fly density in the cage was sufficient, adults were allowed to feed on bovine blood via an artificial membrane feeding system (Hemotek). After about 7 days, the Stomoxys began to lay eggs on moist culture medium. The medium consisted of 900 g wheat bran, 450 g alfalfa meal (ground), 22.5 g dry yeast and 56 g malt. Before adding yeast and malt, the medium was frozen for at least 24 hours to minimize possible feed infestation. Moist culture medium was placed in plastic trays along with the eggs, aerated every two days by stirring and moistened with water using a spray bottle, and moist medium was added as needed. After about 14 days the first larvae pupated. The pupae were collected from the medium and transferred to cages where the adults were allowed to hatch. Prior to the experiment's adults were kept in cages with a 3% glucose solution.

Horse fly (Tabanidae)

Tabanids were chosen, because they have previously been described as mechanical vectors for several pathogens [5]. As we do not have access to laboratory colonies and larval development is relatively long, it was decided to use field collected specimens. As a consequence, different species with different sizes were used.

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2.2.1 Experimental design

2.2.1.1 Arthropods infection protocol

Forty-eight hours before the experiment, arthropods were deprived of sugar to increase the feeding rate. The day before the experiment insects were anesthetized by CO_2 to separate the males from the females in case of mosquitoes and tabanids. Insects were then placed in groups of 1-5 individuals in cardboard box covered with a mosquito-proof net. The paper cups were then placed in a second, sealed containment to prevent the insects from escaping. The lid of the second containment box was punched with holes and sealed with a mosquito-proof net through which blood was fed using blood-soaked cotton pads. The boxes were then transported to the National Reference Laboratory for African Swine Fever (ASF) on the morning of the trial. There, the infectious blood meal was prepared (pig blood spiked with 10 % cell culture virus and 20 µl per ml of blood 5mM ATP as phagostimulant and 1 ml offered to the insects by means of blood-soaked cotton pads (Figure 5). Two experiments with mosquitoes were completed with the fluorescent, cell-culture adapted virus. All other experiments were completed with WSL-cell-culture adapted virus. After feeding (~30 minutes), the arthropods were placed in an incubator in the dark at 10°C, 20°C and 30°C, respectively. Specimens that died during incubation were marked with pins (Figure 6).

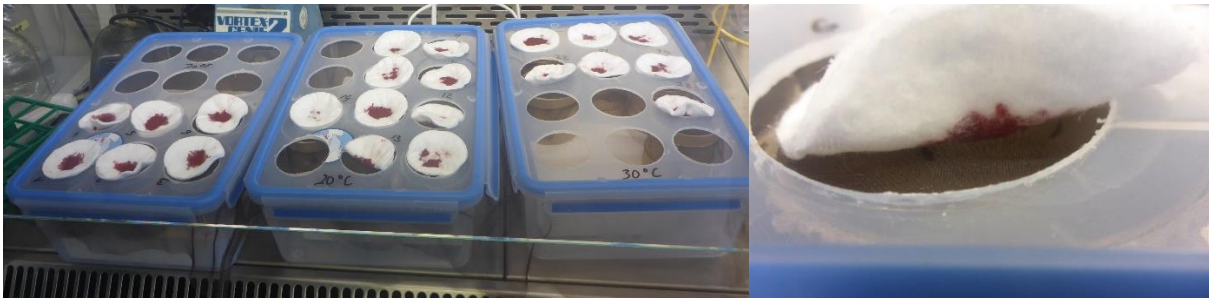

Figure 5: Infection of mosquitoes via blood-soaked cotton pad feeding.

Figure 6: Marking of dead arthropods with pins

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2.2.1.2 Arthropod maintaining and sampling

To maintain the mosquitoes, water-soaked cotton pads were put on top of the boxes and changed daily. Samples were taken after 0 hours, 1 hour, 2 hours, 3 hours, 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. The sampling period was extended to 120 hours, as after the first experiment it was found that ASFV was still detected in the mosquitoes after 72 hours (Table 3). Five experiments (biological replicates) were conducted with at least 50 *Ae. albopictus* in each replicate.

Table 3: Numbers of sampled and tested *Aedes albopictus* at different sampling points (defined by temperature and incubation time)

Temperature	Sampling points									Total
	0 h.p.i	1 h.p.i	2 h.p.i	3 h.p.i	24 h.p.i	48 h.p.i	72 h.p.i	96 h.p.i	120 h.p.i	
10 °C	30	50	50	25	25	25	41	25	15	286
20 °C	25	50	50	49	25	52	56	30	23	360
30 °C	55	25	25	25	33	55	55	24	13	310
Total	110	125	125	99	83	132	152	79	51	956

To maintain *Stomoxys*, sugar-soaked cotton pads (3% glucose-solution) were put on top of the boxes and changed daily. From the second experiment onwards, goat's blood was fed daily after 24 hours, as the first experiment showed that the *Stomoxys* ate each other at 20°C and 30°C, which was probably due to the lack of protein. Samples were taken at 0 hours, 1 hour, 2 hours, 3 hours, 24 hours, 48 hours, 72 hours, 96 hours, 120 hours and 168 hours and at 10°C also at 192 hours, 216 hours, 240 hours and 264 hours (Table 4). Four experiments (biological replicates) with at least 30 *S. calcitrans* in each replicate were conduct.

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Table 4: Numbers of sampled and tested *Stomoxys calcitrans* at different sampling points (defined by temperature and incubation time)

Temperature	Sampling points														Total
	0 h.p.i	1 h.p.i	2 h.p.i	3 h.p.i	24 h.p.i	48 h.p.i	72 h.p.i	96 h.p.i	120 h.p.i	168 h.p.i	192 h.p.i	216 h.p.i	240 h.p.i	264 h.p.i	
10°C	20	20	20	20	20	20	20	16	15	19	10	10	6	6	222
20°C	20	20	20	20	20	20	18	27	24	14	N/S	N/S	N/S	N/S	203
30°C	20	20	20	20	20	30	20	19	19	3	N/S	N/S	N/S	N/S	191
Total	60	60	60	60	60	70	58	62	58	36	10	10	6	6	616

N/S – not sampled

To maintain the tabanids, sugar-soaked cotton pads (3% glucose-solution) were put on top of the boxes and changed daily. Samples were taken at 10°C and 20°C at 0 hours, 72 hours and 96 hours and for 30°C at 0 hours and 72 hours. However, all tabanids that died between 0 and 96 hours were analysed as well (Table 5). We conducted five experiments (biological replicates) with 3-77 tabanids in each replicate, depending on the number of individuals collected in the field.

Table 5: Numbers of sampled and tested Tabanids at different sampling points (defined by temperature and incubation time)

Temperature	Sampling points					Total
	0 h.p.i	24 h.p.i	48 h.p.i	72 h.p.i	96 h.p.i	
10 °C	10	N/S*	N/S	16	4	30
20 °C	11	N/S	1	23	10	45
30 °C	10	1	2	19	N/S	32
Total	31	1	3	58	14	107

* – not sampled

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Two experiments with mosquitoes were completed with the fluorescent, cell culture adapted ASFV strain "ASFV Δ258L_GFPhuCD4" and all other experiments were completed with WSL cell culture adapted ASFV strain "ASFV Armenia".

The overall experimental design is depicted in Figure 7:

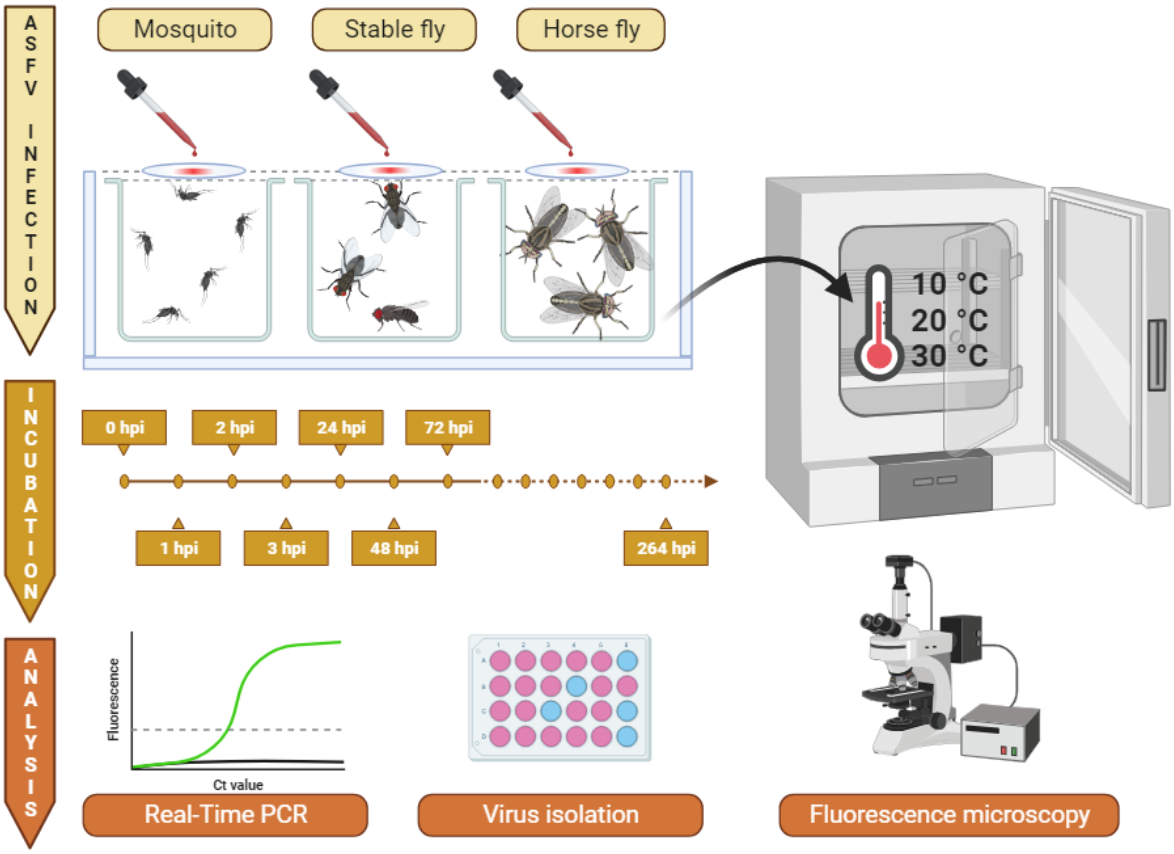

Figure 7: Experimental design to assess the role of mechanical vectors. Insects were fed an infectious blood meal. Thereafter, virus detectability was followed up at different ambient temperatures for up to 264 h. Analyses were done by qPCR, virus isolation and through fluorescence microscopy.

2.3 Possible transmission of ASFV from infected vectors to pigs via oral intake

So far, there is no evidence that blood-sucking arthropods play a significant role in the ASF disease dynamics, but in affected farms, trace amounts of viral DNA could be found in arthropods. In addition, there is experimental evidence that *Stomoxys calcitrans* could transmit the disease via their contaminated mouth pieces or when ingested. Here, it was decided to use *Aedes* mosquitoes as representative for a small arthropod species to add to the data body on mechanical transmission via ingestion of arthropods that have fed on ASFV infected animals. To apply the 3R principle, we conducted a small-scale proof-of-concept study with 6 pigs that were fed 16 fully fed mosquitoes.

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2.3.1 Mosquito infection protocol

Fourty-eight hours before the experiment the sugar solution was removed from the cages. The day before the experiment mosquitoes were anesthetized by CO_2 to separate the males from the females. The pre-selected and sugar-deprived female mosquitoes were placed in groups of 5 mosquitoes in cardboard cups covered with a mosquito-proof net. The cups were then placed in a second, sealed containment to prevent the mosquitoes from escaping. The second containment box was equipped with holes through which blood can be fed using blood-soaked cotton pads. The boxes were then transported to laboratory on the morning of the trial. There, the infectious blood meal was prepared and offered to the mosquitoes by means of blood-soaked cotton pads. After feeding (~2 hours), the mosquitoes were frozen and subsequently fed to pigs as below.

2.3.2 Animal experiment

Six cross-bred domestic pigs (app. 60 kg) were obtained to conduct the study. All animals were moved to the high containment facilities of the Friedrich-Loeffler-Institut (FLI), and kept under appropriate containment and animal welfare conditions. Upon arrival, individuals were ear-tagged and the absence of ASFV-related antibodies and genome was confirmed at the start of the trial. Pigs were fed a commercial pig feed appropriate for their age, mixed with hay cobs and will have *ad libitum* access to water.

At day 0, 16 *Aedes* mosquitoes fully fed on ASFV positive blood were provided to each animal separately in a small amount of feed. Upon feeding the contaminated mosquitoes, the animals were followed up for 21 days to allow serology to be assessed in full. The clinical score system established at the German NRL (see Table 6) for ASF was used to monitor all pigs for clinical signs (at least daily). Rectal temperatures were measured daily as proxy for a possible infection. Blood samples were taken at day 7 and investigated by qPCR. Upon necropsy, blood, spleen and lymph node samples were taken and tested for ASFV genome. Serum was prepared to carry out serological tests (commercial ELISA, i.e. INgezim PPA COMPAC, Gold Standard Diagnostics, and an immuno-peroxidase test).

The study was approved by the responsible animal welfare authority under reference 7221.3-1-044/23.

Table 6: Clinical score system. Clinical signs are assigned score points (+ to +++) based on their severity. Below an example of table for recording this data.

Animal	dpi	Assessment of clinical signs										Temperature °C	Remarks
		was	-	+	++	+++	was	-	+	++	+++		
		Liveliness					Skin						
		Bearing					Eyes						
		Breathing					Faeces						
		Gait					Feed intake						

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2.3.3 Laboratory investigations

Analyses were performed on three independent runs with three technical (diagnostic) replicates each. Following the advice given in the call, real-time PCR and virus isolation (on either permanent wild boar lung cells or peripheral blood monocyte derived primary macrophages) were carried out following accredited standard procedures. Where applicable, pH and temperature were followed (especially for the silage, room temperature was recorded using tracking systems).

For real-time PCR analyses, viral nucleic acids were extracted from all matrices using the NucleoMag VET kit (Macherey-Nagel, Düren, Germany) on the automated KingFisher 96 flex platform (Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturer's recommendations. Subsequently, nucleic acids were analyzed by a WOAH recommended real-time PCR (King et al., 2003) in combination with an internal control detection system (Hoffmann et al., 2006) on a Biorad CFX real-time cycler (Bio-Rad Laboratories, Hercules, USA). In the harmonization phase of the study, additional extraction methods were used as comparators (Plant Pro Kit, Qiagen; Plant Pro seed, Zymo Research). In these cases, the extraction was performed according to the manufacturer's instructions.

For virus isolation of the fluorescent virus ASFVΔ258L_GFPhuCD4 and the cell culture adapted ASFV Armenia WSL adapted P23, permanent wild boar lung cells (WSL, provided by the Collection of Cell Lines in Veterinary Medicine at the FLI, CCLV-RIE 103) were inoculated following standard procedures. Briefly, an initial blind passage was performed in 24-well cell culture plates. For this purpose, WSL cells were pre-grown for 48h, triplicates of each sample were inoculated for 1-2h and afterwards the inoculum was removed and replaced by new medium. For matrices with a low pH another medium exchange was performed after 24h. The plates were incubated for 3-4 days at 37°C and thereafter frozen at -80°C. After a freeze-thaw cycle, the blind passage was inoculated on 96-well plates with pre-grown WSL cells for the final read-out passage and incubated for three days at 37°C. The read-out of the fluorescent virus was done directly with a fluorescence microscope. Regarding the cell culture adapted Armenia strain, cells were heat-fixed/fixated at 80°C for 3h and afterwards an indirect immunofluorescence test (iIFT) was performed. To this means, 96-well plates were washed three times with phosphate buffered saline containing Tween 20 (PBS+Tween 20), blocked with 100 µl phosphate buffered saline with Tween 20 and 5 % horse serum for 1h at 37°C and incubated for 1h with an anti-ASFV p72 antibody (1:750 in PBS+Tween 20, 50 µl per well) at 37°C. Afterwards, plates were washed three times with PBS and incubated for 1h with an AlexaFluor 488 α-rabbit antibody (1:1000 in PBS+Tween 20, 50 µl per well) at room temperature. Subsequently, plates were three times with PBS, 150 µl distilled water was added and plates were read at a fluorescence microscope.

For the haemadsorption test (HAT), i.e. detection of field virus, peripheral blood mononuclear cell (PBMC) derived macrophages were isolated from EDTA-anticoagulated blood obtained from healthy domestic pigs according to published methods. These PBMCs were used for both an initial blind passage of the materials (see above) and final virus titration/detection using standard procedures.

Data analysis, documentation and reports were carried out using GraphPad and, where applicable, SigmaPlot.

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For the overall readout, a worst-case scenario was applied, i.e. the last point of any positivity was recorded, even if negative results lay in-between.

At SVA, total DNA was extracted from 200 µl of matrix elutes on a TANBead Maelstrom 9600 robot with IndiMag Pathogen kit (Indical Bioscience). ASFV DNA was detected by real-time PCR assay with PerfeCTa qPCR ToughMix Low Rox (Quantabio), targeting VP72 gene. The PCR was carried out on an ABI 7500 instrument. A positive control was included in each PCR run and was used to adjust threshold such that this sample had the same Ct value (22) across all PCR runs. The PCR results were exported from 7500 Software v2.3 and summarized using RStudio.

3 Results

3.1 ASFV survival in plant materials

3.1.1 General outcome of study optimization

Based on previous studies, a cell-culture adapted, fluorescent virus was chosen for screening purposes. Such a virus provides the opportunity to read the outcome of *in vitro* experiments without the need of additional staining or processing steps on a robust cell line such as the wild boar lung cell "WSL". However, in the reported experiments, the use of the fluorescent virus had several complications that suggest that this method was not the optimum for the tested setting. Especially with low pH matrices, reading was almost impossible due to high background signals in cultures with slight cytotoxic effects, and recovery rates were low, particularly after filtration (loss of 3 log titres) and with larger sample volumes. To confirm negative results and to provide reliable data sets, studies were repeated on smaller volumes and with high contamination of a cell-culture adapted but non-fluorescent virus (see methodology), where possible. Within the timeframe of the project, silage production and grass harvest were not possible to be repeated and for that reason, re-contamination trials were conducted using mature silage.

Matrix phenomena were also seen with qPCR and for this reason, different, partly specialized extraction kits were used for plant-based samples. After considering the results, however, the routine method for ASF samples was re-instated. The results leading to this action are depicted in the overview below.

Table 7: Triplicates in columns 2 to 4 were extracted with the NucleoMag Vet Kit (Macherey-Nagel), triplicates in colums 6 to 8 with the Plant Pro Kit (Qiagen), and columns 10 to 12 with the Plant Pro seed (Zymo Research). Results are presented as cycle threshold values in qPCR.

	1	2	3	4	5	6	7	8	9	10	11	12
A		28,05	26,78	27,67		39,17	N/A	N/A				N/A
B		28,19	27,49	30,03		N/A	38,03	N/A		33,83		
C		28,5	25,93	27,19		39,01	38,24	N/A		33,28		
D		27,03	26,21	25,37		39,4	38,09	N/A		33,36		
E		25,97	26,79	27,34		39,42	37,81	N/A		33,62		
F		27,24	25,5	26,99		N/A	39,02	39,05		32,72		
G		25,47	27,05	24,56		37,88	N/A	36,44				
H		27,7	27,32	28,35		37,04	N/A	38,27				31

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In the stability trials in arthropods, complete blood meals were not always visible. For this reason, a cytochrome B detection [8] was included that showed that the arthropod had taken up porcine blood. Only arthropods that showed a considerable uptake of blood were included in the further ASF studies.

The overall experience led to addition of several small amendments such as additional centrifugation of samples prior to extraction and virus isolation, re-use of filtration for cell culture isolation, change of media after 24 h incubation, and use of higher amounts of antibiotics in the media. The final protocols are as above.

3.1.2 Analysis of chemical composition and fermentation characteristics of grass and corn silages

The analysis of CON silages was accomplished by an accredited (DIN EN ISO/IEC 17025) and certified (DIN EN ISO 9001) German laboratory. Analyses of contents of DM, crude ash (CA), crude protein (CP), crude fiber (CF), crude fat (CL), true protein (TP), sugar, starch, enzyme-soluble organic matter (ELOS), utilizable crude protein (uCP), acid detergent fiber (ADF) and neutral detergent fiber (NDF) were conducted by NIRS according to methods of VDLUFA Volume III A. 31.1-31.3, 2004. Determination of the short chain fatty acids (SCFA, C2-C6) and alcohols (ethanol, propanol, butanol) as well as lactic acid (LAC) were performed using gas chromatography. The NH_3-N content were analysed colorimetrically according to von Lengerken and Zimmerman (1991).

The ASFV-free silages (CON) and ASFV-spiked silages (ASFV1, ASFV2) were stored identically at different storage locations (CON at BfR, Berlin and ASFV1/ASFV2 at FLI, Riems) due to biosafety requirements regarding ASFV contaminated material. The CON silages were examined for their nutrient content and fermentation parameters to evaluate ensiling quality of ASFV1/ASFV2 silages. An overview about target values of a good silage, are provided in table 7, and were used for ensiling success evaluation.

Table 8: Target values for quality parameters of good grass and corn silages (compiled from Galler 2011)

Quality parameter	Grass silage (range of tolerance)	Maize/corn silage (target value)
Dry matter (g/kg OM)	300-400	280-350
Particle length (cm)	2-4	0.5
CA (g/kg DM)	< 100	3.5-4.5
CP (g/kg DM)	150-180	70-90
CF (g/kg DM)	220-260	170-210
Sugar (%)	2-4	
pH	4,4-4,8	< 4.5
LAC (g/kg DM)	25	> 20
Acetic acid (g/kg DM)	< 20	> 10

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Butyric acid (g/kg DM)	< 3
NH_3-N (%)	< 10
Yeast (1.000/g Feed)	< 100
Clostridia (1.000/g Feed)	< 10
Molds (1.000/g Feed)	< 10

Fermentation characteristics in grass silage (CON) are shown in Figure 8. The average dry matter of grass silages slightly decreased from 282 ± 3.6 g/kg original matter (OM) at day 3 to 274 ± 4.9 g/kg OM at day 180 of trial at storage ambient temperature of 10°C (p = 0.083). Storage temperature conditions of 20°C or higher (37°C) showed no significant change of dry matter during fermentation from 274.5 ± 1.7 g/kg OM (day 3) to 281.9 ± 10.8 g/kg OM (day 90, $p = 0.351$) and 284.2 ± 6.3 g/kg OM (day 3) to 286.8 ± 2.4 g/kg OM (day 28, p = 0.534), respectively (Figure 8A). Dry matter loss was overall very low, indicating very good ensiling properties and low CO_2 production. Moreover, optimal pH value (4.0 – 4.8) was measured in grass silages at all time-points and storage conditions (Figure 8B). Results of lactic acid content (Figure 8C) show the higher the level of lactic acid in silages, the lower the pH values (see also Figure 10A). It was concluded that the ensiling process took place under favorable conditions, as lactic acid was formed quickly, and lowered the pH value in the silages sufficiently. These results are confirmed by sufficiently amounts of acetic acid during storage duration (Figure D) and the simultaneously very low production of butyric acid during storage (10°C: 3.3 to 1,6 g/kg OM; 20°C: 2.2 to 1.7 g/kg OM; 37°C: 2.2 to 1.2 g/kg OM, Figure E).

High levels of butyric acid are usually a result of malfermentation in the presence of clostridia, which, alongside with nutrient losses, leads to sensory impairments in the silage. Another indicator of malfermentation is the formation of NH_3-N as a result of increased proteolysis and deamination by certain groups of bacteria under high pH conditions. However, percentage of NH_3-N in grass silages was very low between 3.5% to 4.4%. The formation of alcohols, primarily ethanol, indicate the presence of yeasts in the silages, which degrade sugars into alcohols under anaerobic conditions. In general, alcohol formation during ensiling was low. The results of the percentage of alcohol in the silages showed increasing levels during 180 days of ensiling at 10°C of storage temperature (0.40-0.62%, p = 0.035) and was about factor 1.8 higher in silages stored at 20°C until day 90 (0.71-0.79%, p = 0.322). At storage temperatures of 37°C a decrease in the formation of alcohols was shown from day 7 of storage duration (0.81-0.45%, $p < 0.001$). No other (sensory) signs for impaired quality of the grass silages was determined.

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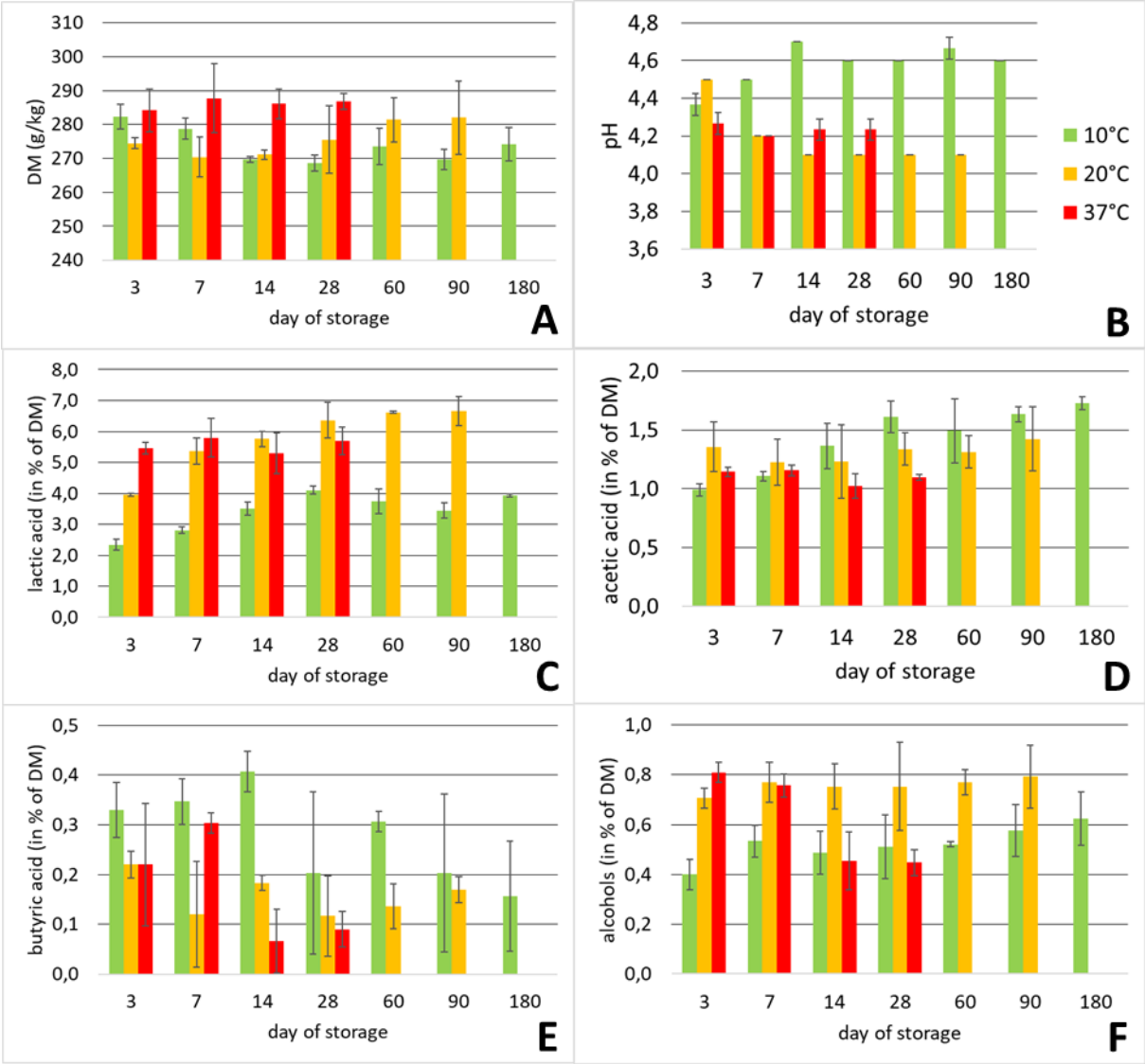

Figure 8 Dry matter (DM) and fermentation parameters in grass silage treated with homofermentative lactobacilli (3 x 10^5 cfu/kg) and molasses (30g/kg) after storage at 10°C (green bars), 20°C (yellow bars) or 37°C (red bars) for up to 180 days of silaging.

Fermentation characteristics in corn silage (CON) are shown in Figure 9. At storage condition of 4°C, no change in dry matter content (p = 0.183) as well as very low formation of butyric acid (p = 0.759) and alcohols (p = 0.058) was found during 180 days of ensiling. Those effects were attributed to limited microbial activity due to the low-temperature storage conditions. The pH of 4.8 at the beginning of the trial was higher than the target value for maize silage but reached an optimum (< 4.5) at day 180 of storage duration (Figure 9B). Also, lactic acid formation was low at 4°C. Concomitantly with the low content of other undesirable fermentation parameters (e.g. butyric acid < 3 g/kg DM, sum of alcohols < 8 g/kg DM), it can be concluded that maize silage of very good quality was produced under the 4°C low-temperature storage conditions. As the temperature increases during storage, fermentation parameters hint towards increased microbial activity in the silages. This is

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reflected in a greater loss of dry matter (10°C: 410 ± 3.2 g/kg OM to 379 ± 13.3 g/kg OM, $p = 0.017$; 20°C: 399.2 ± 10.4 g/kg OM to 383.5 ± 1.2 g/kg OM, $p = 0.138$), while pH significantly decreased (10°C: $p = 0.004$; 20°C: $p = 0.010$) and lactic acid formation increased (10°C: $p = 0.002$; 20°C: $p = 0.037$) during 180 days at 10°C or 90 days at 20°C of storage conditions, respectively. The good correlation ($R^2 = 0.868$) of the reduction in pH value with a simultaneous increase in lactic acid formation in maize silage is shown in Figure 10B. Statistical analysis indicates significant formation of acetic acid ($p = 0.002$) and alcohols ($p < 0.001$) but constantly low levels of butyric acid ($p = 0.547$) during ensiling at 10°C for 180 days. Storage of maize silage at 20°C showed high formation of lactic acid of 25 to 35 g/kg DM during 90 days of ensiling (Figure 9C). Despite increasing levels of acetic acid (day 90: 9 to 28 g/kg DM, Figure 9C) and alcohols (da 90: 6 to 14 g/kg DM, Figure 9D), no signs of deterioration were observed, which rather points towards shift in activity of major bacterial groups in the silages with increasing storage temperature. In addition, a low contamination by clostridia is confirmed by the consistently low butyric acid content (≤ 1 g/kg DM, Figure 9F). Overall, the evaluation of fermentation parameters indicate that the laboratory scale silages of grass and maize were of good quality and comparable to silages used for feed in practice.

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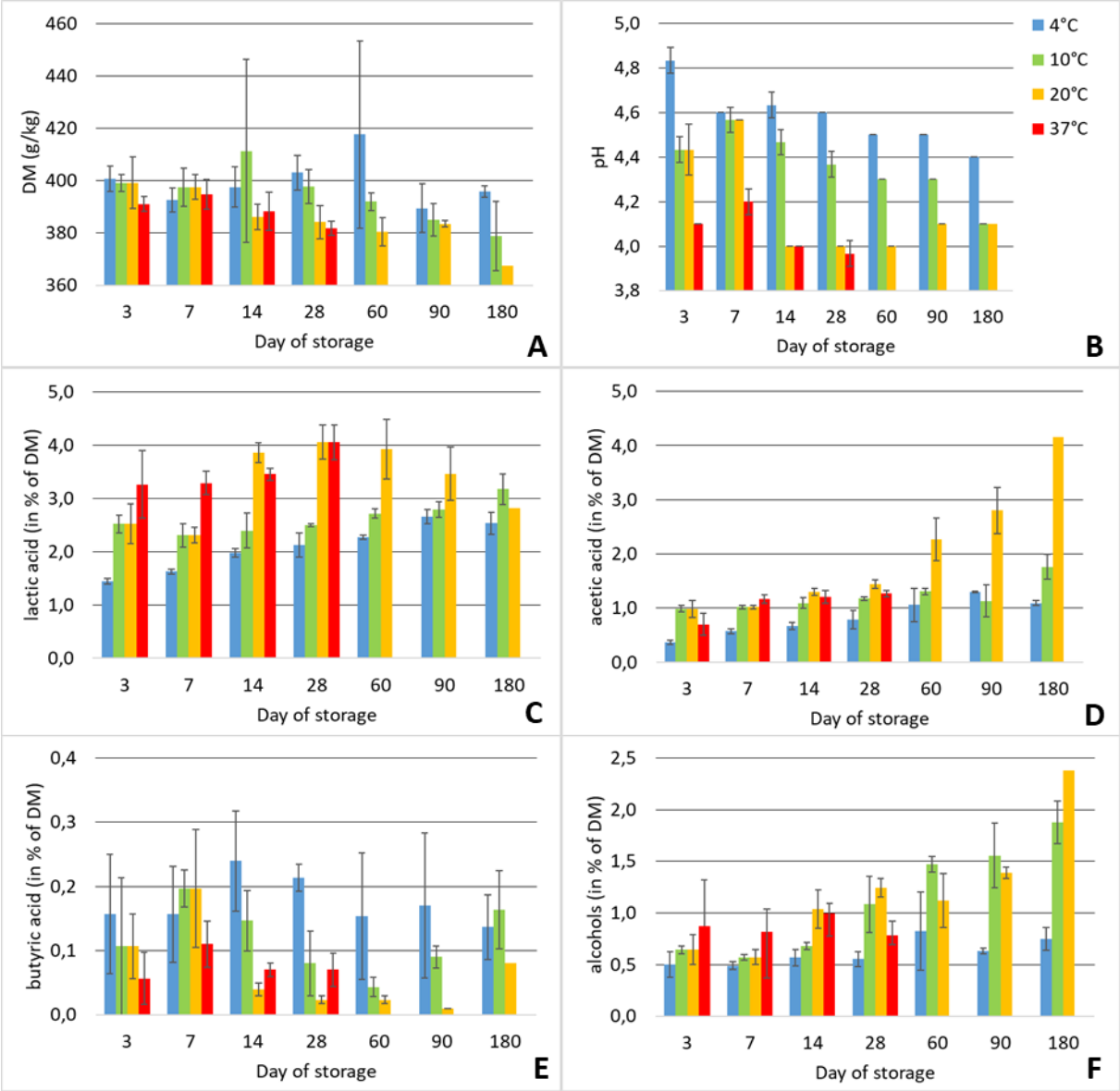

Figure 9 Dry matter (DM) and fermentation parameters in maize silage treated with homofermentative lactobacilli (3 x 105 cfu/kg) and molasses (30g/kg) after storage at 4°C (blue bars), 10°C (green bars), 20°C (yellow bars) or 37°C (red bars) for up to 180 days.

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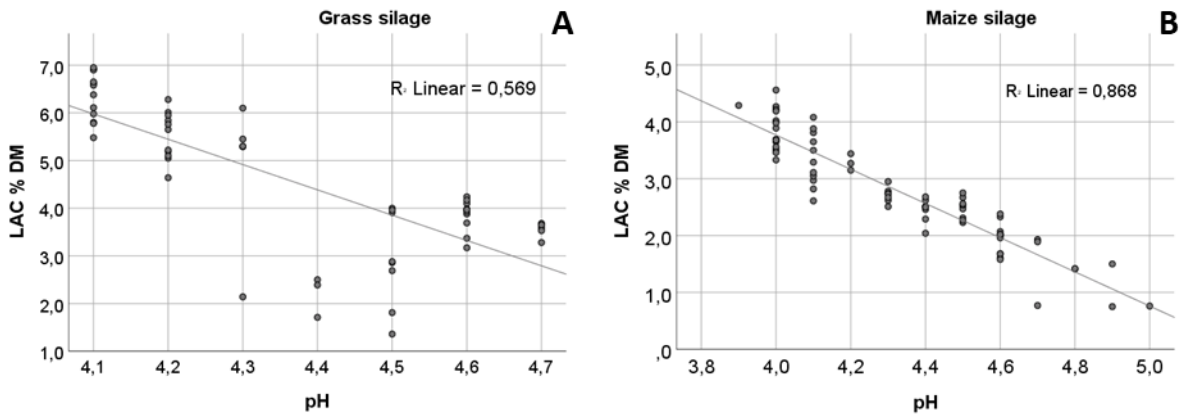

Figure 10 Correlation of pH value and lactic acid content (%) in grass silage (A) and maize/corn silage (B)

3.1.3 Stability of ASFV in grass and corn/maize silage

ASFV genome was generally detectable in all contaminated silages over the entire study period (180 days), and irrespective of the storage temperature. However, some problems were seen with retrieval of the virus and its DNA, especially with the fluorescent virus. Infectious virus was not detected, even in samples directly frozen after the ensilaging (e.g. 3-4 h after contamination).

When doing the heavy re-contamination of grass silage with the cell culture adapted virus without fluorescence marker, infectious virus was recovered up to seven hours while genome was detected over the entire duration (Figure 11).

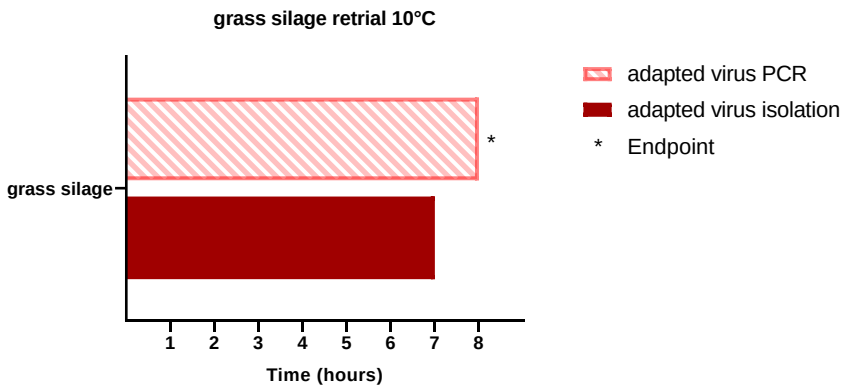

Figure 11: Detection of viral genome and infectious virus after contamination of a mature grass silage with the cell culture adapted ASFV strain.

The results confirm, even with all its limitations resulting from technical obstacles, that silage does not allow viral persistence for longer periods of time. Inhibitory effects are seen even

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with viral genome detection. The overall results are depicted in the overview figure 13 below (together with the other matrices), raw data can be found in the annexes.

3.1.4 Stability of ASFV on cut grass

When frozen directly after preparation, viral genome could be detected on grass over the entire study period. Despite that, no infectious virus was detected in this matrix. At 10°C and 20°C, genome was detectable only at early or very early time-points. When using high loads of field virus, detection of viral genome was possible up to 7 days post contamination. No infectious virus was detected at any time and with any modifications of the protocol.

Some limitations were observed in this study part, including the retrieval of the contaminant from a larger volume of grass and, the influence of the organic compounds affecting the cell culture. However, as no virus was detected at any time-point, the overall risk is probably low. Worst-case scenario results are presented in the overview figure 13 below and raw data can be found in the annexes.

3.1.5 Stability of ASFV on beet and potato

Viral genome and field virus contamination was detectable over the entire study periods of 180 (beet) and 274 (potato) days post contamination at -20°C. Problems were again seen with the fluorescent virus that was very difficult to read with these matrices. At 4°C, viral genome was again detectable over the entire period of 180 and 274 days. Under these conditions, field virus was successfully re-isolated up to 120 days on beet and 28 days on potato. For the field virus, only representative time-points, i.e. 7 and 28 days, were tested, and for this reason, one should rather assume a longer stability (comparable to beet). On beet, fluorescent virus was also detectable at early time-points (up to day 3). Representative results are depicted in Figure 12 and in the overview figure 13 below. Raw data can be found in the annexes.

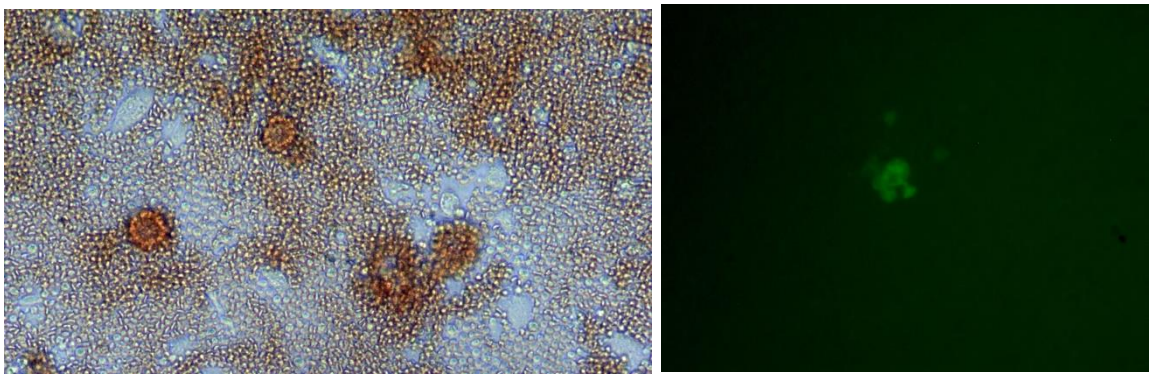

Figure 12: Representative results for beet kept at 4°C for 28 days (HAT) and 3 days (fluorescence)

Due to common harvesting and storing practices, only potatoes were tested at higher temperatures. At 10°C, viral genome was again detected over the whole study period. Infectious field virus was detectable at low levels up to day 28. The lower titre of the fluorescent virus was not to be re-isolated. As field virus was only tested at days 7 and 28, a longer stability cannot be ruled out. However, the titre decreases over time and at day 28, only single cells were infected when reading the plate.

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At 20°C, the study period was 90 days. Viral genome could be detected over the entire period whereas only field virus was detectable up to 7 days (no detection at later time-points).

At 37°C, the study lasted 28 days and only viral genome was detectable (up to the end of the study part).

All data are summarized in the overview figure 13 below.

3.1.6 Stability of ASFV on wheat treated with propionic acid

Treated wheat was heavily contaminated and kept as proof-of-concept at 10°C, 20°C, and 37°C. While viral genome was detected over the entire study period of 60 days, infectious field virus was found up to day 7 at both 10 and 20°C (and in the sample taken at time-point 0 that was kept at –20 °C over 60 days). Virus isolation was not successful with the fluorescent virus at any time-point. Given that the contamination dose was much lower, the outcome indicates that the viral load is decreasing quickly. An overview can be found in the overview figure 13 below; the aggregated raw data can be found in the annexes.

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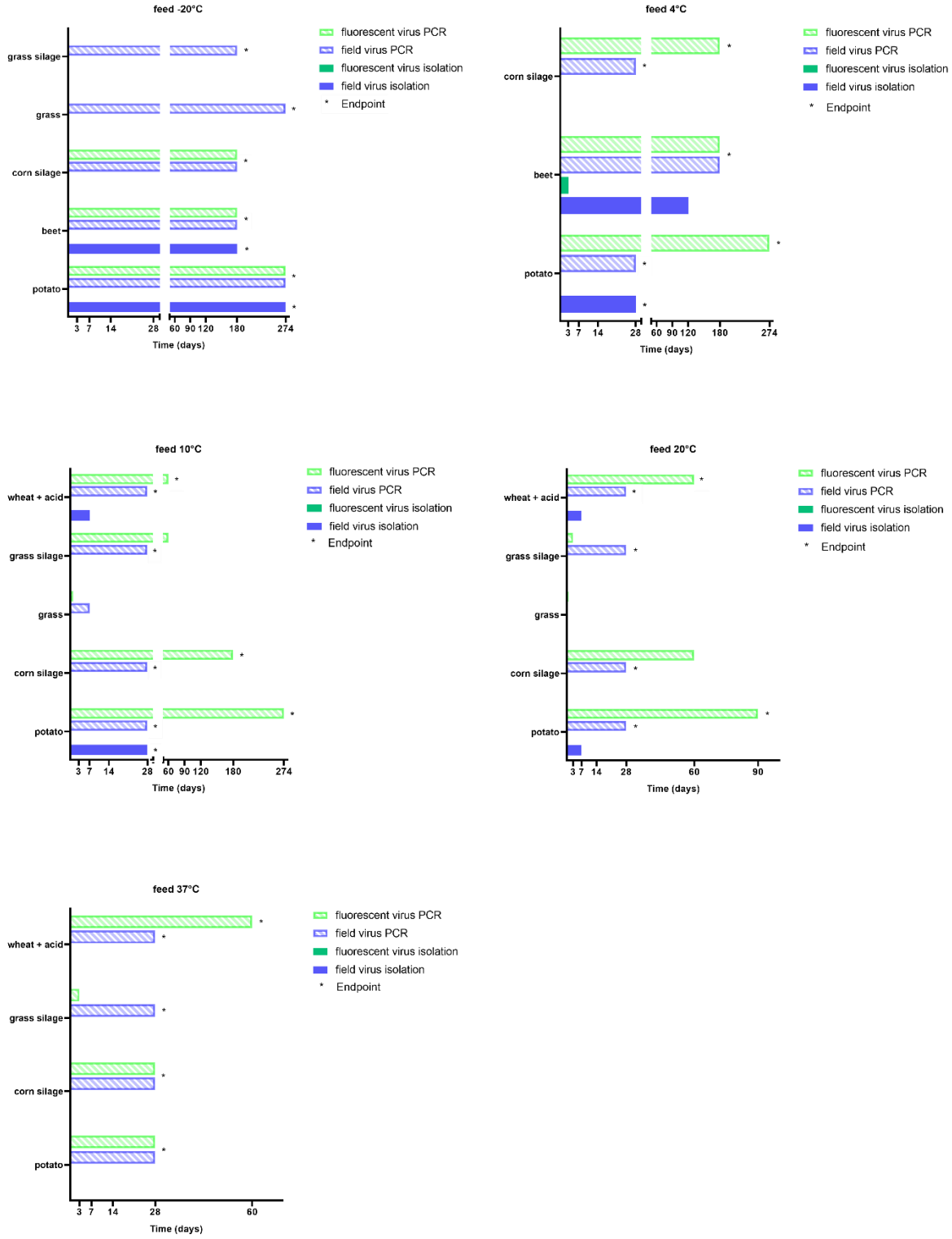

Figure 13: Stability of ASFV and genome on feed

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3.1.7 Stability of ASFV on roughage and bedding

African swine fever virus genome was detectable over the entire study period (274, 90 or 28 days depending on the temperature) on bedding matrices kept at all temperatures. At -20°C, virus detection was only possible on bark till the end of the trial at day 274. Matrix effects and low pH may have influenced the viability of the virus from the beginning. There are, however, also effects from retrieval of material from the contaminated bedding/roughage material. Repetitions with high titre viruses at other temperatures showed positive detections.

In detail, field virus was detectable at 4°C on hay, peat, and saw dust for 7 days, on bark for 28 days. On peat, limited replication was seen also till 7 days post contamination.

At 10°C, infectious field virus was only detected on bark at 7 days post contamination. No other re-isolations were possible at higher temperatures.

The results are depicted in figure 14 below.

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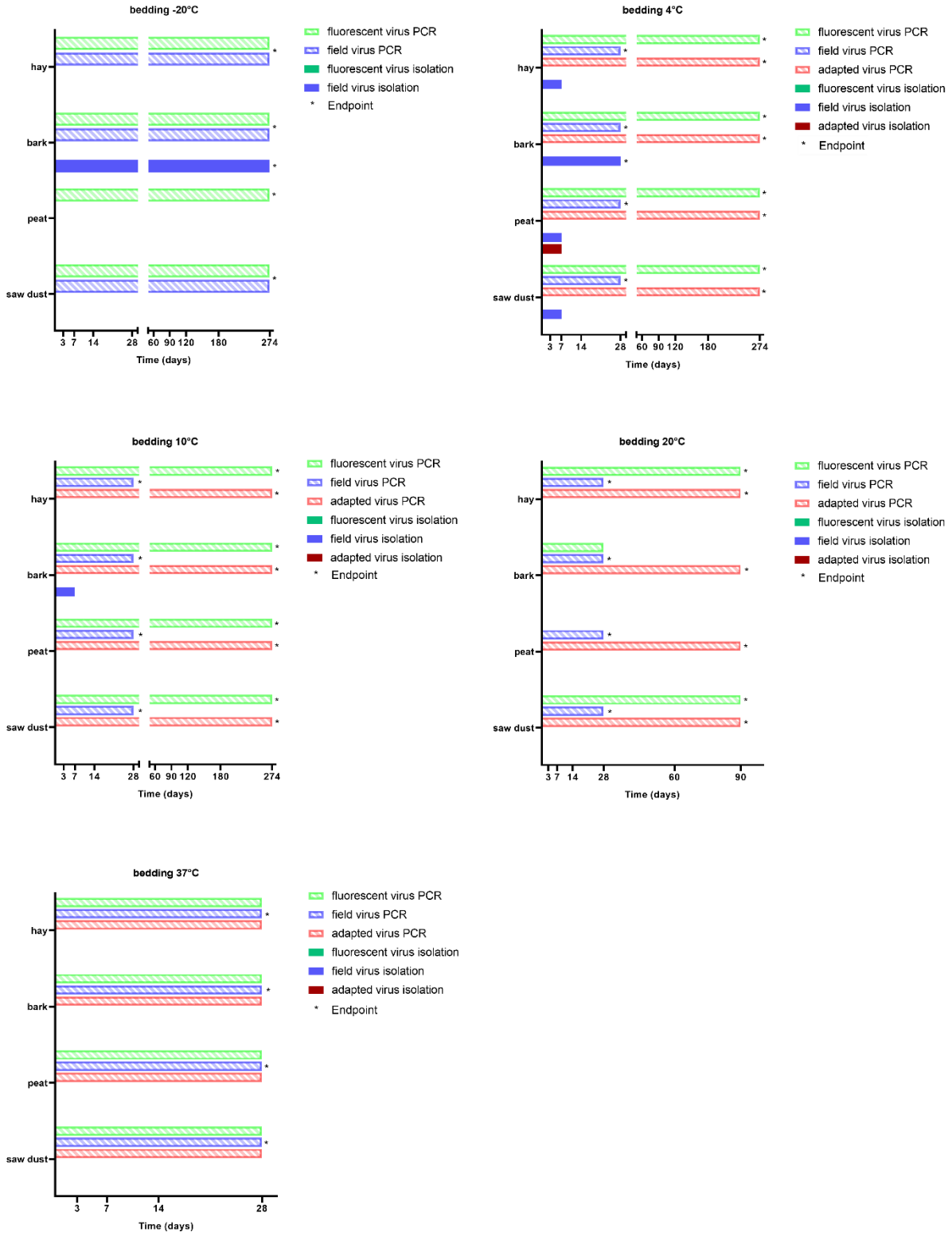

Figure 14: Stability of ASFV on roughage and bedding materials

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3.1.8 Stability of ASFV on barley, oats, rapeseed, straw and wheat

African swine fever virus DNA was detected in almost all samples under the experimental conditions reflecting unsurprisingly a high genetic stability. Propionic acid (1%) treatment decreased viral DNA in wheat by 5.5 ~6 Ct regardless of the temperature and incubation time whereas no effect was observed in straw samples. The propionic acid had close contact with the viruses spiked in wheat therefore destroying some viruses. By contrast, the acid sprayed into the straws might not contact the viruses as did in wheat. The amount of virus sprayed into straws was larger than that in wheat. Therefore, the observed difference was mainly a technical issue, e.g., virus-acid ratio, efficiency of mixing of viruses and acid in different matrices, spaces, etc.

The outcome of qPCR is depicted in figures 15 and 16.

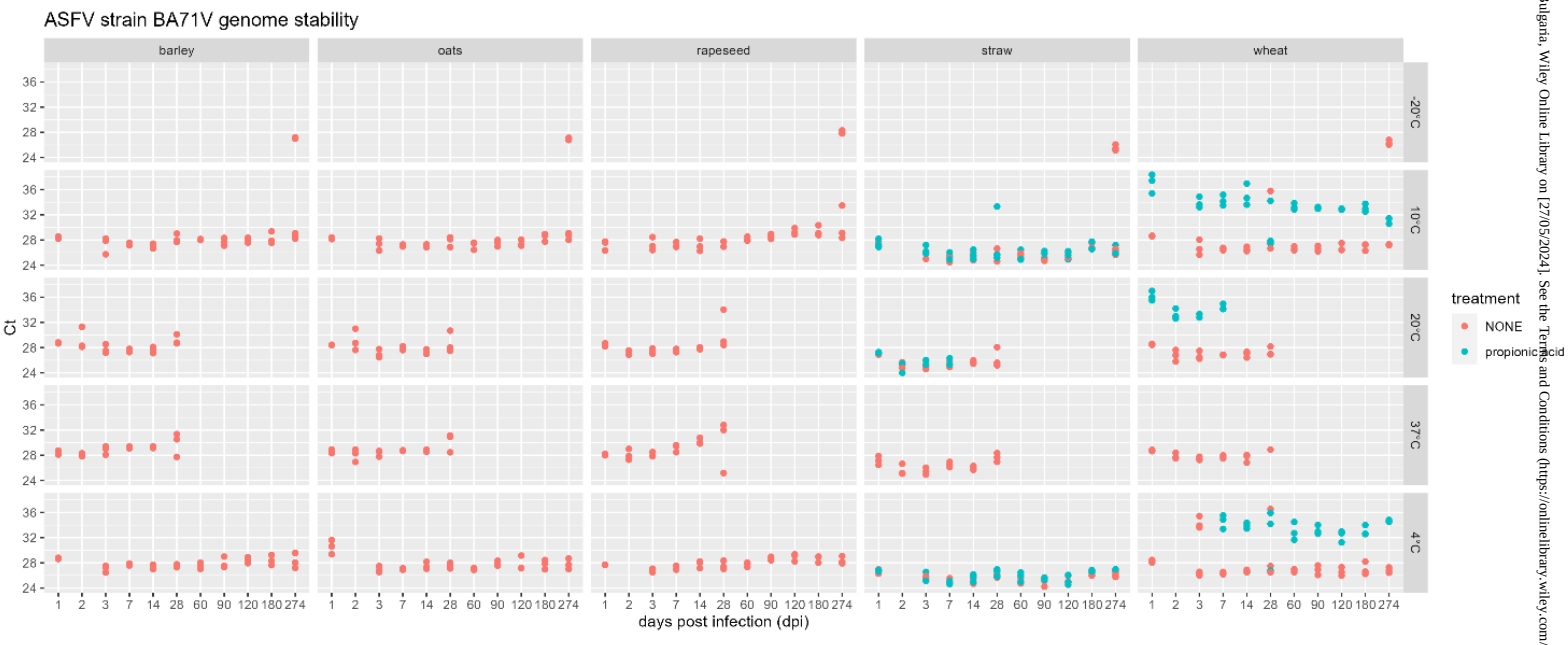

Figure 15: Detection of BA71 viral DNA over time.

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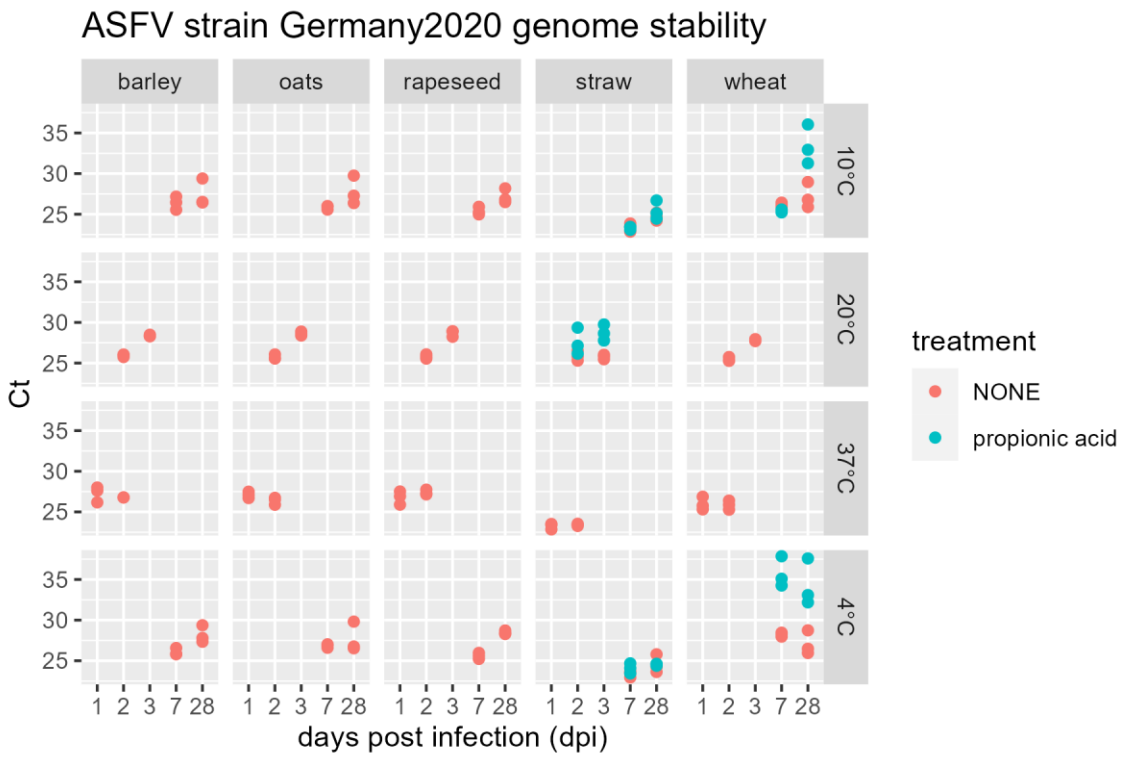

Figure 16: Detection of field ASF viral DNA over time.

Infectious cell culture adapted virus was only detected for t0 samples of barley and straw, and at one day post contamination in single samples of oats, wheat, and barley kept at temperatures up to 20°C (no detections at 37°C). Field virus was detected up to day 3 on untreated wheat and straw samples kept at temperatures up to 20°C. No virus detections were made on rapeseed.

3.1.9 Stability of ASFV in arthropods upon feeding infectious blood meals

The outcome of the experiments with mosquitoes (*Ae. albopictus*) is depicted in Figure 17. Only mosquitoes that showed sufficient blood uptake were included in the study.

It can be seen that digestion of the blood meal occurred in a temperature dependent manner. While no viral genome was detectable after 72 h at 30°C, genome was detectable at 20°C for 72 h and at 10°C for 120 h.

Infectious virus was detected at 20 and 30 °C only at early time-points, i.e. up to 3 h, at 10°C, however, virus was re-isolated also at 120 h (5 days).

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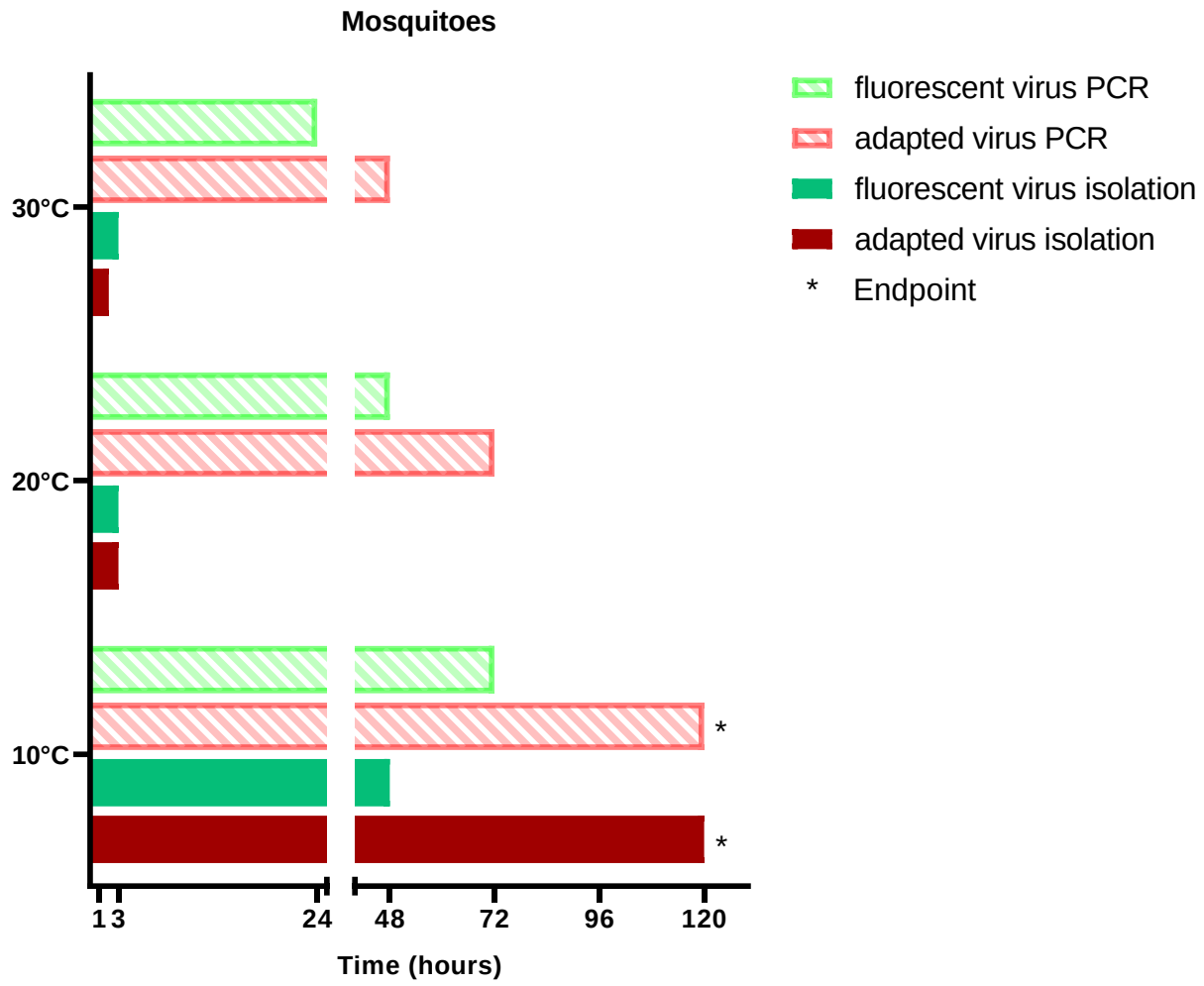

Figure 17: Stability of ASFV in mosquitoes.

In the experiments with Stomoxys, detectability was much longer, agreeing with previous studies [10]. At 30°C, viral genome was detected up to 48 h, infectious virus for 24 h. At 20°C, viral genome was still detectable at 72 h, virus for 48 h. Surprisingly, viral genome was detectable for at least 264 h at 10°C. At this temperature, infectious virus was still found at 168 h (7 days) post feeding. An overview is presented in Figure 18.

Catches of tabanids were rather rare and their blood uptake was very limited (55 tabanids were weak positive for pig cytochrome B). No virus was re-isolated from any of the tabanids and viral genome detection was scarce (one tabanid only). Based on the data that could be generated in this short project, the role of tabanids remains rather unclear. However, if they would play a major role, detections should have been possible.

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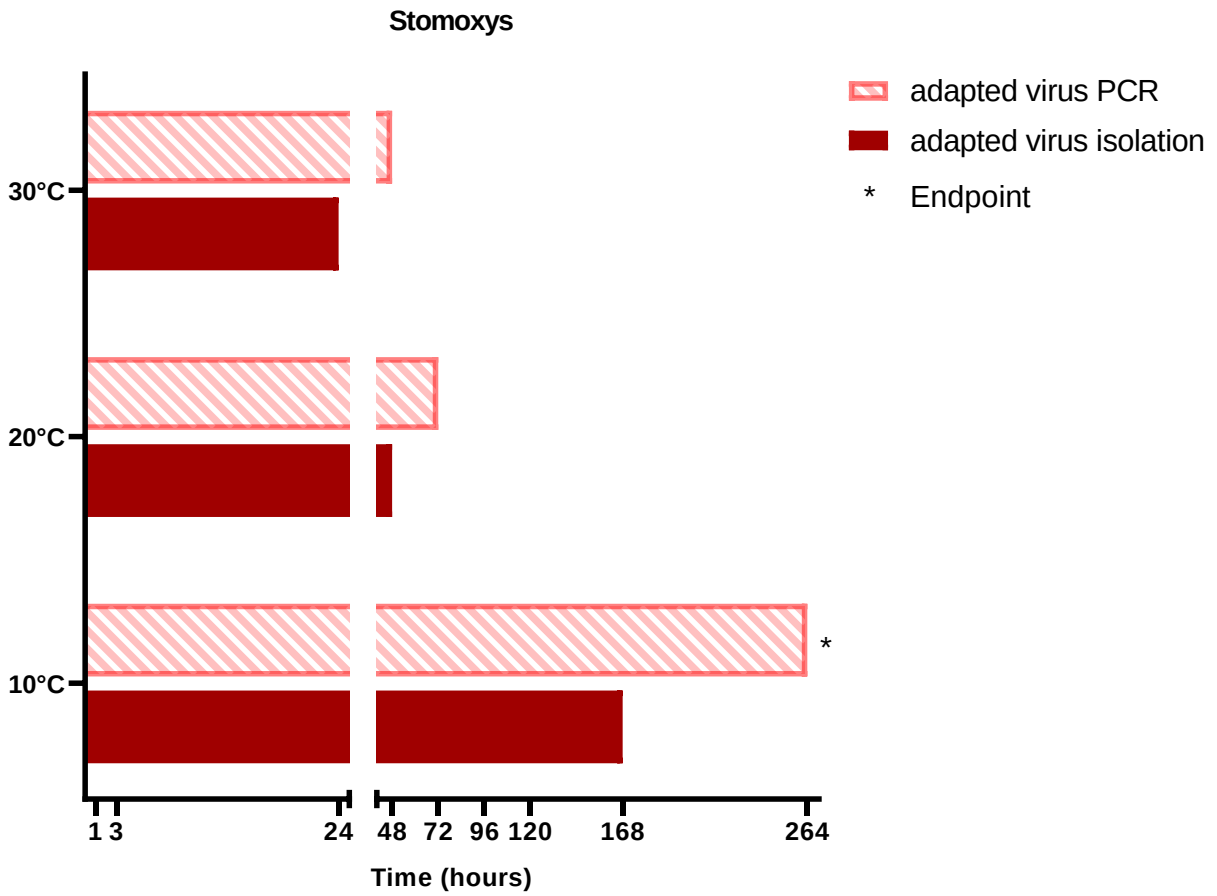

Figure 18: Stability of ASFV in Stomoxys.

3.1.10 Outcome of the feeding experiment

Upon feeding 6 fattening pigs with 16 Aedes mosquitoes that had previously been fed with ASFV positive blood, none of the pigs became infected or seroconverted. The mosquitoes were positive for virus nevertheless. This is probably a matter of chance and repetition that a low amount suffices for infection.

4 Conclusions

Feed and bedding

The study clearly shows that methodology is a critical issue when conducting stability testing. Established systems using primary cells provide the most sensitive results, despite technical hurdles, and are therefore preferable. However, well-characterised, adapted viruses can also be used, but these need to be optimised with the appropriate matrices and procedures.

The stability of the ASF viral genomes is expected to be very high and it is therefore not surprising that the viral genome can be detected over a wide temperature range and long

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periods of time. The detection of genomes from silage, grass and hay is difficult. These problems could only be partially overcome by adapted extraction methods. We assume that these effects still indicate very rapid inactivation.

The situation is different for the detection of the infectious virus. Despite several attempts, the virus could only be detected in a few samples and then only at cool temperatures. Surprisingly often, the virus was detected in beet and potatoes.

Similar results were obtained with bedding material. Detection of the virus is limited to cool storage temperatures and short periods of time. At 4°C, inactivation usually occurs after 7 days, with the exception of one bark sample that remained weakly positive after 28 days. Even at 10°C, only the bark allowed a single detection after 7 days. Storage at moderate temperatures was always suitable for inactivating the virus under the experimental conditions.

Treatment with propionic acid had little additional effect, which correlates with the generally low detectability.

Arthropods

The studies on the detectability of the virus in arthropods show that the virus is generally detectable for a certain period of time depending on temperature and volume. As expected, the virus is detectable in *Stomoxys* for a relatively long time, at 10°C even significantly longer than expected, i.e. over 168 hours. At cool temperatures, the infectious virus can also be detected in mosquitoes for up to 120 hours.

The feeding experiment did not lead to infection of pigs. However, the power of this proof of concept study is limited.

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APPENDIX A– Supplementary data

All supplementary data is available in zenodo: https://doi.org/10.5281/zenodo.10973175

A.1. Feed and bedding raw data (.xlsx)

A.2. Feed and bedding retrial (.xlsx)

A.3. Arthropod studies raw data (.xlsx)

A.4. Supplementary analyses arthropods (.pdf)

A.5. Photo documentation (.pdf)

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