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Article

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Metagenomic assessment of airborne and surface viral signals and worker acceptability during a partial-lid intervention in a Cambodian live bird market

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ABSTRACT

Mechanical defeathering can disperse viral material in live poultry markets, but interventional machine modifications must fit routine work. This nonrandomized field study evaluated partial lids on defeathering machines at Orussey Market, Cambodia, across six sampling days, three with lids and three without. Targeted RT-qPCR and target-enriched metagenomics characterised viral RNA in air samples collected during a three-hour air-sampling period and in nearby surface samples collected before and after that period, while work questionnaires assessed usability. Sequencing detected 73 poultry-associated viral taxa. Influenza A virus, avian coronavirus and rotavirus G together accounted for over 80% of relative metagenomic abundance in 14 of 18 air samples. In air samples, RT-qPCR cycle thresholds correlated inversely with metagenomic relative abundance for avian influenza virus and Newcastle disease virus (Spearman's $\rho = -0.645$ and -0.684, respectively). Airborne richness was descriptively lower on days with lids, but targeted viral detections did not change consistently, and composition profiles overlapped. Surface profiles changed from before to after the air-sampling period on days with and without lids; the study did not establish a lid-associated difference in these changes. All five workers reported discomfort and perceived slower work; none wanted to use the lid in its tested form, although three would continue if it were permanently integrated into the machine. With only three sampling days per condition, the study did not establish a consistent reduction in airborne or surface viral contamination associated with lid use. Future studies should evaluate lids redesigned with workers alongside complementary measures addressing ventilation, hygiene, and clean water and waste management to reduce occupational exposure.

Keywords: environmental virome; live poultry markets; environmental contamination; airborne viral material; surface contamination; poultry processing; intervention evaluation; worker acceptability

INTRODUCTION

Traditional food markets play an important role in food supply, livelihoods, and community life. Markets selling and slaughtering live poultry provide fresh food and income while bringing birds, workers, and customers into close contact, creating opportunities for exposure to zoonotic pathogens during handling and processing [1, 2]. Reducing exposure to these pathogens is a public health priority, but preventive measures must also be compatible with routine market activities. A public health intervention that complicates processing or is difficult to maintain may be poorly suited to sustained use, even when effective, or when workers understand the intended protective purpose.

The live poultry market environment changes as animals enter, held, and are processed. Secretions, feces, carcass wash water, and processing waste can carry pathogens into shared spaces, while working practices influence their movement and removal[1, 3]. In 2025, Cambodia reported more laboratory-confirmed human influenza A virus (H5N1) cases to the World Health Organization than any other country [4]. In Cambodian live bird markets (LBMs), multiple avian influenza virus (AIV) subtypes including H5, H7, and H9 have been detected in poultry and carcass wash water, and investigations have documented extensive AIV circulation and environmental contamination [5-7]. Viable H5N1 and H9N2 viruses have also been isolated from personal air samplers worn by market workers [8]. Therefore, practical and acceptable interventions are needed to reduce potential exposure to these and other pathogens circulating in these markets.

Mechanical defeathering removes feathers from scalded poultry carcasses using an open-topped machine in which a rotating base moves the carcasses against rubber fingers. This process can disperse particles carrying avian influenza viruses into the market environment when infected poultry are processed. These machines generate respirable particles, and experimental processing of influenza-infected poultry has shown release of infectious virus into the air [9, 10]. Material released during processing may remain airborne or reach nearby surfaces through deposition. Surfaces can also become contaminated through splashing and contact during routine work [1, 3]. Sampling air and surfaces can therefore broaden an intervention assessment, although

surface detections alone cannot distinguish airborne deposition from contamination through splashing or direct contact.

Partial lids have been proposed as a simple modification to reduce material escaping through the defeathering machine opening. In China, computational modeling estimated a reduction of more than 65% in aerosol escape, while field measurements showed a 57% reduction in airborne poultry genetic material at worker breathing height; a reduction in airborne AIV RNA was not demonstrated [11]. Controlled experiments in Bangladesh found lower concentrations of airborne particulate matter <2.5 μm in diameter ($PM_{2.5}$) with fully covered machines but did not directly assess AIV detection[12]. Therefore, studies are needed to determine whether lids affect viral RNA detection in air and on nearby surfaces.

Combining air and surface sampling with metagenomics allows assessment of whether partial lids on defeathering machines change the viral RNA detected in the air and on nearby surfaces and allow comparison with the wider market virome [3, 13]. This study evaluated the impact of partial lids on defeathering machines during routine poultry processing at Orussey Market in Phnom Penh, Cambodia. Targeted RT-qPCR and metagenomics were used to assess viral RNA in air and surface samples collected with and without lids. Worker acceptability was also assessed to determine whether the design could be used in everyday market practice.

RESULTS

Poultry-associated viruses were detected in air and on nearby surfaces

Sampling of poultry, air, and nearby surfaces during routine poultry processing provided biological and operational contexts for comparing intervention conditions. Four market stalls next to each other were sampled across six sampling dates, with all four machines operating with partial lids on three dates and without lids on three dates. The 924 collected specimens yielded 72 analytical samples: 18 poultry pools, 18 air samples, and 36 surface pools comprising samples collected before or after the three-hour air-sampling period. Sampling days, rather than individual stalls, provided the three independent replicates per group.

Using target-enriched metagenomics, 73 poultry-associated viral taxa were identified across the samples (Fig. 1). Here, “taxa” denotes distinct taxonomic assignments represented as features in the filtered viral read-count table. Avian coronavirus (Gammacoronavirus galli) and influenza A virus were prominent in poultry and air samples, occurring across most dates and analytical samples Other detected taxa included rotavirus A (Rotavirus alphagastroenteritidis), rotavirus D (Rotavirus deltagastroenteritidis), rotavirus F (Rotavirus phigastroenteritidis), rotavirus G (Rotavirus gammagastroenteritidis), avian orthoreovirus (Orthoreovirus avis), avian leukosis virus (Alpharetrovirus avileu), chicken astrovirus, and avian metapneumovirus (Metapneumovirus avis) (Fig. 1b). The environmental measurements therefore captured a range of poultry-associated viruses alongside the three RT-qPCR targets: AIV, Newcastle disease virus (NDV), and infectious bronchitis virus (IBV).

Poultry RT-qPCR results described part of the viral background in the processing environment. AIV Ct values ≤40 from 16 of the 18 poultry pools were included in the analysis, with daily medians ranging from 22.51 to 32.91. NDV was detected in eight samples. Pool size and bird-species composition varied between samples, so these results characterize the sampled birds as a whole, rather than individual-level prevalence.

Airborne RT-qPCR comparisons showed no significant differences between days with and without partial lids

Because use of the partial lid was intended to limit the distribution of material during defeathering, RT-qPCR was used to test 18 air samples for the three predefined viruses: AIV, NDV, and IBV. Daily median Ct values were used for the primary comparison so that the analysis retained the sampling day as the intervention unit (Supplementary Fig. S1; Supplementary Table S2). For AIV, the median Ct value of the daily medians was 32.49 with partial lids, and 31.78 without lids – a difference of +0.71 Ct. Corresponding values were 33.46 and 33.24 for NDV (+0.22 Ct), and 24.65 and 25.95 for IBV (−1.30 Ct). Differences were calculated as with-lid minus without-lid values. The Ct differences between lid conditions were positive for AIV and NDV and negative for IBV, and none of the comparisons was statistically significant after Benjamini–Hochberg correction (Supplementary Table S2).

Secondary comparisons of individual air samples also resulted in no statistically significant differences (Supplementary Table S2). Median AIV Ct values were 33.49 with lids (range 29.77–37.02; n = 9) and 32.33 without lids (30.90–37.40; n = 8). NDV medians were 33.57 (29.48–37.52; n = 9) and 33.24 (27.22–37.14; n = 9), respectively; IBV medians were 25.26 (23.45–28.13; n = 9) and 25.74 (24.07–27.70; n = 7). These exploratory sample-level tests did not account for within-day clustering. Eligible numeric results were available from 9/9 samples collected with lids and 8/9 without lids for AIV, 9/9 under each group for NDV, and 9/9 with lids and 7/9 without lids for IBV. These counts meet the numerical-analysis criteria; they are not confirmed positivity rates. The comparisons could not establish equivalence or exclude a meaningful lid effect.

Airborne viral richness was descriptively lower with lids, while community profiles overlapped

Probe-based enriched metagenomics were used to examine whether lid use was accompanied by broader changes in the detected airborne virome. Observed viral taxon richness, defined as the number of taxonomic features detected per sample after filtering, was higher in air samples than in poultry pools in both the intervention and non-intervention groups (Fig. 2b). On the three days with lids, daily median viral richness values (ranges across the three air samples) were 23 (23–28), 26 (24–31), and 23 (19–26), compared with 28 (24–28), 31 (25–32), and 30 (27–30) on the three days without lids, respectively (exact two-sided day-level P = 0.10). Mean daily counts were 24.8 taxa with lids and 28.3 taxa without, a descriptive reduction in taxon richness of 12.5%. The mixed-effects sensitivity analysis estimated 3.56 fewer taxa with lids (95% CI −7.74 to 0.63; P = 0.078).

This descriptive difference in richness was not accompanied by clear separation in diversity or community composition. Simpson diversity did not differ significantly between air samples collected with and without lids, with similar findings for Chao1 and Shannon indices (Fig. 2a; Supplementary Fig. S2). Exploratory differences between matched poultry and air Simpson values were larger on days with lids, but group comparison was inconclusive (P = 0.077; Fig. 2c).

Bray–Curtis PCoA showed substantial overlap between air samples collected with and without lids (Fig. 3a). In the pairwise PERMANOVA using whole-day permutations, lid use accounted for 3.0%

of the variation in the two-group comparison (R^2 = 0.030; exact P = 0.900; Benjamini–Hochberg-adjusted q = 0.900; Supplementary Data 1). Jaccard dissimilarity produced a similar ordination pattern (Supplementary Fig. S3). These compositional analyses do not measure absolute airborne viral concentrations. Exploratory distances from the poultry reference centroid were greater for air samples collected with lids than without, but the comparison was inconclusive (P = 0.070; Fig. 3b). This analysis examined differences between sampled poultry and airborne profiles rather than directly quantifying containment. Figure 3c summarizes the direction of taxon-specific differences in relative abundance between poultry and air samples and between air samples collected with and without lids.

Across all 153 pairwise comparisons among the 18 air samples, avian coronavirus produced the largest mean absolute change in Bray–Curtis distance when removed. Its negative mean signed contribution indicated that its inclusion generally increased the similarity between air samples. Together, influenza A virus, avian coronavirus, and rotavirus G accounted for more than 80% of relative abundance in 14 of 18 air samples. The leave-one-taxon-out analyses described variation across the air dataset rather than the contributions of individual taxa to differences between lid intervention conditions (Supplementary Fig. S4; Supplementary Data 2). Comparisons of daily median metagenomic relative abundance, expressed as reads per million (RPM), identified no statistically significant differences between lid conditions for AIV, NDV, or the broader avian coronavirus assignment (Supplementary Table S2).

Targeted PCR and sequencing revealed similar variation for AIV and NDV

To help interpret the environmental measurements, eligible RT-qPCR Ct values were compared with matched metagenomic RPM across both lid groups. This comparison assessed the extent to which the two methods reflected similar patterns of variation; it was not intended to provide a separate estimate of the lid effect. In air samples, Ct values were inversely associated with corresponding metagenomic RPM for AIV and NDV, whereas the association between IBV Ct values and avian coronavirus RPM was weak. Zero RPM values were retained.

In air samples, RT-qPCR Ct values showed significant inverse associations with corresponding metagenomic RPM for AIV and NDV, whereas no evidence of an association was observed for

IBV. In air samples, AIV M-gene Ct values were inversely associated with RPM assigned to *Alphainfluenzavirus influenzae* (ρ = −0.645, 95% CI −0.863 to −0.223; P = 0.0064; FDR-adjusted q = 0.0095; n = 17). NDV Ct values were similarly associated with *Orthoavulavirus javaense* RPM (ρ = −0.684, 95% CI −0.876 to −0.305; P = 0.0018; q = 0.0053; n = 18). The association between IBV Ct values and the broader avian coronavirus assignment was weak (ρ = −0.181, 95% CI −0.631 to 0.360; P = 0.4995; q = 0.4995; n = 16; Fig. 4).

In poultry pools, AIV RT-qPCR Ct values were also inversely associated with corresponding metagenomic RPM (ρ = −0.9000, 95% CI −0.9662 to −0.7224; P = 6.55×10^{-6}; q = 3.27×10^{-5}; n = 16). The poultry NDV association did not meet the FDR-adjusted threshold (ρ = −0.7638; P = 0.0357; q = 0.0595; n = 8). For surface samples collected before the air-sampling period, AIV correlations were not estimable because all three retained pairs had RPM = 0; the NDV correlation relied on three pairs (ρ = −1.0000). For surface samples collected after the air-sampling period, correlations were ρ = −0.4956 for AIV (95% CI −0.8096 to 0.0391; P = 0.0631; q = 0.0788; n = 15) and ρ = −0.7665 for NDV (P = 0.0324; q = 0.0595; n = 8). Only poultry AIV met q < 0.05 among the estimable poultry and surface comparisons (Supplementary Fig. S5; Supplementary Table S4). For IBV, targeted RT-qPCR Ct values were compared with metagenomic RPM assigned to avian coronavirus (*Gammacoronavirus galli*), the species that includes IBV. The measurements were obtained from separate RNA extractions using different kits. These methodological differences may have influenced the observed Ct–RPM association. Correlation estimates, confidence intervals, and significance tests did not account for clustering within sampling dates.

Surface viral profiles changed over the air-sampling period with and without lids

Surface sampling was used to examine a second environmental outcome: whether viral profiles on plastic sheets near the defeathering machines changed during the three-hour air-sampling period, and whether profiles from samples collected after the air-sampling period differed between days with and without lids. Sheets above the machines and on nearby walls were sampled before and after the air-sampling period; swabs were pooled by date, analytical unit, and collection time point (before or after the air-sampling period). These samples captured surface contamination during routine work, rather than airborne deposition alone.

Targeted RT-qPCR was performed on plastic-sheet samples collected before and after the three-hour air-sampling period. AIV and NDV were analysed in both pooled and individual swab samples, whereas IBV was analysed only in individual swabs. Among individual swabs, AIV results meeting the Ct ≤40 criterion increased from 4/36 before the air-sampling period to 14/36 after the air-sampling period on lid days, compared with 0/36 and18/36 on no-lid days. Corresponding numbers of individual swab samples meeting the Ct ≤40 criterion were 1/36 to 11/36 and 7/36 to 17/36 for NDV, and 32/36 to 33/36 and 30/36 to 35/36 for IBV. Median Ct values in samples collected after the air-sampling period with and without lids were 38.15 and 38.43 for AIV, 38.40 and 37.25 for NDV, and 33.03 and 33.24 for IBV, respectively. Pool-level AIV and NDV results, together with Ct medians and ranges for all targets in samples collected before and after the air-sampling period, are provided in Supplementary Table S6. Although detection counts in samples collected after the air-sampling period were numerically lower on lid days for all three targets, these descriptive findings do not establish a consistent reduction in surface viral signals attributable to the intervention.

Observed richness and Simpson diversity were generally higher in surface pools comprising samples collected after versus before the air-sampling period under both conditions (Fig. 2a; Supplementary Fig. S6). Avian coronavirus was detected in seven of 18 pools of samples collected before the air-sampling period and all 18 pools of samples collected afterward, whereas rotavirus G was detected in every surface pool. Therefore, detection of some taxa became more widespread over the air-sampling period against a background of other taxa detected throughout.

Paired PERMANOVA comparisons identified changes in community composition from before to after the air-sampling period both on days without lids ($R^2 = 0.191$; exact P = 0.0195; adjusted q = 0.0342) and days with lids ($R^2 = 0.237$; exact P = 0.0156; adjusted q = 0.0298). Surface samples collected at the end of the three-hour air-sampling period did not show statistically significant separation by lid use ($R^2 = 0.048$; exact P = 0.800; adjusted q = 0.884; Supplementary Data 1). These comparisons establish within-group temporal differences in the detected communities but they do not test whether the magnitude of change differed between groups.

The change in plastic-surface virus composition over the three-hour air-sampling period was then compared between lid and no-lid days. For each day and stall, the Bray-Curtis dissimilarity indices were compared between the paired plastic-sheet samples collected before and after the air-sampling period. The magnitude of these within-pair distances did not differ between lid and no-lid days (p = 0.80, Supplementary Fig. S7). Direct comparison of the multivariate before-to-after community shifts between lid conditions revealed no evidence that the shifts differed (p = 0.90; Supplementary Fig. S8). At the taxon level, no significant differences were detected between lid and no-lid days when samples collected before and after the air-sampling period were analysed separately after false discovery rate correction (Supplementary Fig. S9). Separate paired analyses within each lid condition likewise identified no significant before-to-after changes after correction. Although 26 taxa showed positive estimated changes on no-lid days and zero or negative changes on lid days, these patterns were not statistically significant (Supplementary Fig. S10). These findings suggest that the observed before-to-after changes were not detectably associated with lid status; however, this comparison was based on only three independent sampling days per condition.

To describe which taxa most influenced variation across the surface dataset, contributions were examined across all 630 pairs among the 36 samples. Rotavirus G produced the largest mean absolute change in Bray–Curtis distance when removed and contributed overall to similarity. Avian coronavirus had the second-largest mean absolute contribution and contributed overall to dissimilarity. Rotavirus G also ranked first in the Jaccard analysis (Supplementary Fig. S11; Supplementary Data 2). These descriptive outputs concern the full surface dataset rather than a specific lid contrast.

Processing activity and local environmental conditions varied across sampling dates

Recorded poultry-processing activity varied across the six sampling dates, with daily counts ranging from 357 to 673 chickens and ducks during the three-hour air-sampling periods (Supplementary Table S3). Recorded counts per three-hour air-sampling period were 539 birds on partial-lid days and 577 on no-lid days.

Across the six sampling dates, 129 sets of temperature, relative humidity, and local air-velocity measurements were recorded across the four sampled stalls (Supplementary Table S5). Temperature ranged from 28.5 to 42.7 °C, relative humidity from 39.4 to 83.6%, and local air velocity from 0 to 3.5 m/s. Daily median temperatures ranged from 31.60 to 35.30 °C, and daily median relative humidity from 58.10 to 72.50%. Local air velocity was recorded as zero in 84 of 129 measurements.

Descriptive comparisons showed that processing activity and airborne viral measurements did not follow a uniform pattern across sampling dates. The highest recorded throughput occurred on 6 October (673 birds), which also had the highest daily median AIV and avian coronavirus metagenomic RPM values (Supplementary Table S2). However, the highest NDV RPM was recorded on 7 October, when 607 birds were processed. On 16 September, the daily median NDV RPM was zero despite 587 birds being processed.

Surface RT-qPCR results showed more frequent Ct-eligible detections after the air-sampling period under both intervention conditions (Supplementary Table S6). These observations, together with the surface metagenomic findings, describe viral signals during routine market activities but do not establish whether processing activity or local environmental conditions accounted for their variation.

Perceived protection did not translate into support for routine lid use

Before using the lid, all five workers reported the belief that breathing market air could cause illness; four were concerned or slightly concerned about handling poultry, and four were willing to try the lid. All five workers anticipated discomfort, four expected slower work, two anticipated difficulty cleaning the lid, and two anticipated excess heat (Table 1). After use, three of five workers agreed with the statement, “Using the partial lid makes me feel safer during poultry processing.” Three workers believed that the lid could reduce airborne feathers, dust, or germs. However, four found it difficult or very difficult to use; all five selected discomfort and perceived slower work as reasons they might avoid the lid, and only one was satisfied. All five answered “No” to the direct question about wanting to use the partial lid, whereas three stated that they would continue using

295 it if it became a permanent part of the machine. Two workers suggested enlarging the opening,
296 and two would recommend the lid to workers in other markets.

297 Before lid use, all five workers reported having received information or training on illness prevention
298 and obtaining water from the market piped-water supply. All five reported cleaning the defeathering
299 machine “after each use”. Three described soap-based cleaning methods, one reported using
300 water alone, and one described another cleaning method in an open-ended response.

301 In the adapted 10-item System Usability Scale (SUS) section, all five workers agreed that the lid
302 was too complicated; four disagreed that it was easy to use, and three agreed that technical
303 assistance was needed. Four reported feeling confident using the lid. In contrast to the responses
304 to the direct yes/no question, three agreed with the adapted SUS statement that they would like to
305 use the lid frequently (Supplementary Table S7). These findings provide descriptive feedback from
306 the five participating workers on usability, willingness to use the lid, and potential design
307 improvements. They reflect initial experience with the tested configuration and do not assess
308 sustained use during routine full-day operations.

309 **Table 1**. Worker perceptions and acceptability before and after partial lid use (n = 5).

Domain	Before use	After use
Perceived protection and safety	5/5 believed breathing market air could cause illness; 4/5 were concerned or slightly concerned about handling poultry. 3/5 believed a lid could reduce feathers, dust or germs.	3/5 believed the lid could reduce feathers, dust or germs; 3/5 felt safer; 1/5 agreed it made the environment cleaner and more comfortable.
Usability and workflow	5/5 anticipated discomfort; 4/5 slower work; 2/5 difficult cleaning; 2/5 excess heat. No worker reported an existing machine cover or barrier.	4/5 found the lid difficult or very difficult to use; 5/5 selected discomfort and 5/5 slower work as barriers; 1/5 selected difficulty cleaning.
Willingness to use	4/5 were willing to try the partial lid.	5/5 answered “No” to the direct question about wanting to use the partial lid; 3/5 would continue using it if it became a permanent part of the machine; 2/5 would recommend it to other workers.
Routine practices	5/5 answered yes when asked whether they clean the machine “after each use”; 3/5 wore a mask always/often; 1/5 always wore gloves.	Not assessed.
Suggested improvements	One worker suggested gas rather than wood for boiling/scalding water.	2/5 suggested enlarging the lid opening.

DISCUSSION

Reducing zoonotic disease risks in LBMs requires improvements that preserve market functions and fit the realities of daily market work. Biosafety and biosecurity guidelines offer numerous recommendations, but the evidence supporting their effectiveness under routine operating conditions remains uneven [1, 2, 14]. A recent scoping review of traditional food markets identified interacting biological, structural, and behavioral risks and found that the reported effects of interventions varied across settings and outcomes [14]. Translating recommendations into meaningful improvements requires testing interventions where they will be used, measuring what they change, and understanding whether workers can incorporate them into their practices and daily routines.

Partial lids for poultry defeathering machines offered a practical opportunity to examine these requirements together. The intervention targeted one processing activity within a market where poultry, workers, water, equipment, and contaminated materials continually interact. Airborne viral richness was descriptively lower with intervention, but targeted RT-qPCR did not demonstrate consistent reductions in AIV, NDV, or IBV, and airborne viral community profiles overlapped. Surface viral detections and community profiles changed over the air-sampling period both with and without intervention, without clear separation of profiles from samples collected after the air-sampling period by lid use. Worker responses identified perceived benefits alongside substantial operational barriers. Together, these findings suggest a possible influence on one aspect of airborne contamination, but do not demonstrate a consistent environmental benefit or an acceptable configuration for routine use.

The findings from direct poultry sampling provide essential context for interpreting both air and surface measurements. AIV was detected at Ct values ≤40 in 16/18 poultry pools, confirming that processing occurred against a background of viral circulation. Because pool size and species composition varied, these results characterize the sampled material rather than individual-bird prevalence or a standardized measure of infection pressure. Earlier Cambodian market surveillance studies have similarly documented extensive AIV circulation in poultry and processing environments [5-7]. During previous surveillance efforts in Orussey market, AIV was detected in

39.6% of sampled chickens, 51.3% of ducks, and 93.1% of carcass wash water samples, with co-circulation of H5, H7, and H9 viruses [5]. These differences illustrate why environmental detection cannot be interpreted as a direct estimate of infection prevalence among birds. Environmental samples can integrate material from multiple animals and activities, while poultry swabs characterize the birds and anatomical sites sampled. A recent Cambodian metagenomic surveillance study also recovered poultry-associated viruses from environmental samples that were missed by concurrent bird sampling [13]. Agreement between poultry and environmental findings therefore supports virus circulation, while differences between them need not represent conflicting evidence.

The airborne richness pattern deserves further investigation because every daily median with the lid was lower than every daily median without intervention. Nevertheless, the comparison remained inconclusive. Richness describes the number of detected taxa, rather than their total quantity, infectivity, or contribution to human exposure. A reduction in the number of detectable taxa can therefore coexist with continued detection of dominant viruses and broadly overlapping community profiles. The lack of a consistent response in the targeted assays further limits interpretation as a general reduction in airborne viral contamination. Metagenomics broadens the assessment beyond a small set of predefined targets, but the additional information must be interpreted alongside targeted measurements rather than treated as a substitute for quantitative exposure assessment [3, 13] .

Previous intervention studies help explain the importance of these distinctions. Wei et al. reported that a partial lid reduced airborne avian 18S rRNA concentrations by 57% in the worker's breathing zone [11]. However, infection varied among the processed chickens, complicating comparisons of airborne influenza virus RNA. Effects also differed by sampling position. Recent experiments in Bangladesh demonstrated reductions in fine particulate matter with some modifications to slaughtering and defeathering equipment, using $PM_{2.5}$ as a proxy for potentially virus-containing aerosols [12]. These findings support the physical rationale for modifying processing equipment, but do not establish equivalent reductions in specific viruses across an operating market. The present study extends the comparison through targeted viral assays, metagenomic profiles,

surface sampling, and worker feedback. The mixed findings reinforce that reductions in particles, avian material, viral richness, and infectious exposure are related objectives that require distinct evidence.

The results of the surface sampling demonstrate why evaluating only air samples may provide an incomplete assessment of viral contamination in the market environment. Pooled AIV detection increased from 3/18 to 15/18 surface pools between samples collected before and after the air-sampling period, including 12 pairs that changed from not detected to detected. Pooled NDV detection increased from 3/18 to 8/18 pools. Individual plastic-sheet swabs showed corresponding increases, from 4/72 to 32/72 samples for AIV and from 8/72 to 28/72 for NDV. Avian coronavirus detection also became more widespread in surface pools, increasing from 7/18 before the air-sampling period to 18/18 afterward. Together with the community analyses, these observations are consistent with accumulation of detectable viral material on exposed surfaces during processing. They do not establish the absolute quantity deposited by processing or from the air, nor do they distinguish infectious material from residual nucleic acids. The surface findings therefore identify continuing contamination during work without establishing that the use of a partial lid altered the rate or extent.

Several mechanisms could contribute to surface contamination. Airborne deposition is one possibility, but splashing water, direct contact with poultry and equipment, handling, waste, and movement through the processing area could also redistribute material [1, 7, 13, 15, 16]. Environmental sampling in Egyptian LBMs similarly detected influenza A virus RNA in water, surface and air samples [17].The exposed plastic sheets sampled the complete working environment rather than emissions from the defeathering machine alone. No machine cleaning was observed during the three-hour air-sampling period sand carcass wash water was used in the machines without the addition of clean water during the air-sampling period. These observations support evaluating surface hygiene and water management alongside equipment modifications, although they do not identify which practice produced the observed viral profiles. They also highlight a questionnaire limitation: all workers reported cleaning the machine “after each use,” but

the undefined term “use” could refer to a processing cycle or the working day as no cleaning was observed during the air-sampling period.

Processing volume could also influence both airborne contamination and surface deposition. Recorded throughput ranged from 357 to 673 chickens and ducks during the three-hour air-sampling periods. However, the date- and unit-level observations did not support a simple relationship in which processing more birds invariably produced greater airborne viral signals. Surface detection gains also occurred across dates and operating conditions. Throughput measures activity, but does not establish the proportion of infected birds, the rate of viral shedding, or the contribution of surrounding work to viral deposition. Cambodian surveillance studies have associated peak AIV circulation with several major festivals, including Lunar New Year [6, 18]. Such periods may involve changes in poultry demand, movement, sourcing, and processing, but the impact of those processes should not be treated as interchangeable. Earlier studies in Hong Kong identified poultry sales and species-related differences as relevant to virus isolation patterns [19]. The present observations support considering activity and infection pressure together; they do not establish that throughput or festival-associated changes explained the findings, or that these factors had no influence.

Temperature, humidity, and local air movement may further influence how viral material is transported, deposited, and retained. The 129 recorded environmental measurements described substantial variation, with temperatures of 28.5–42.7°C, relative humidity of 39.4–83.6%, and local air velocities of 0–3.5 m/s. Most velocity readings were zero, but these point measurements cannot characterize overall ventilation or air exchange. Previous studies on the effectiveness of partial-lids found differences between modeled and field dispersion patterns associated with background ventilation [11]. Experimental influenza virus studies also demonstrate that environmental effects depend on the system and biological material being examined: humidity-associated losses of infectivity in simulated cough aerosols contrast with sustained infectivity across humidities when virus was suspended with airway-derived material [20, 21]. These studies provide mechanistic context rather than direct estimates for the diverse viruses and matrices in this study. The environmental records are therefore useful for describing sampling conditions and generating

explanations for variability, but do not establish independent effects on either air or surface outcomes.

The wider intervention literature indicates that other measures address processes beyond the reach of a defeathering lid. Market structure, poultry holding, turnover, and continuing introduction of birds can sustain viral circulation independently of dispersion during processing [22, 23]. In Hong Kong, rest days combining poultry removal with cleaning and disinfection produced immediate reductions in AIV isolation, although effects varied over time and between poultry groups [19, 24] . A second monthly rest day did not produce a statistically significant additional reduction after adjustment for other factors [19]. Subsequently, preventing overnight poultry storage was associated with an additional 84% reduction in AIV isolation from chickens relative to the preceding period with two monthly rest days [25]. These findings support measures that interrupt infection continuity within markets but concern a particular regulatory system. They do not establish the same effect in Cambodia or rank poultry-management measures against lids in a direct comparison. Rather, they illustrate why reducing viral circulation in birds and reducing dispersion during slaughter are complementary objectives.

Environmental hygiene addresses a different part of the problem and is particularly relevant to the surface findings. Work on local air extraction and mechanical barriers during poultry evisceration also demonstrates that engineering approaches extend beyond covering one processing machine, although findings from industrial processing cannot be transferred directly to LBMs. In Guangzhou, market closure combined with cleaning and disinfection was associated with reductions of 79% in H7N9 RNA detection and 92% in virus isolation in retail markets [26]. These findings demonstrate that broader measures can reduce environmental contamination but cannot identify the independent contribution of disinfection during continued processing. Conversely, an observational study in Bangladesh found that reported monthly cleaning appeared protective, while disinfection practices were not otherwise associated with environmental influenza virus detection [27]. This does not establish that disinfection is ineffective; it illustrates the limitations of broad practice categories and the need to assess implementation and subsequent recontamination. Work on local air extraction and mechanical barriers during poultry evisceration also demonstrates that

engineering approaches extend beyond covering one processing machine, although findings from industrial processing cannot be transferred directly to LBMs [28]. Environmental surveillance in live poultry markets in Changsha found that operating exhaust systems were associated with lower AIV positivity [29]. This association supports evaluating ventilation alongside equipment modifications. For Orussey market, the relevant question is which feasible combination of equipment, hygiene, water, waste, and workflow measures produces sustained improvements in both air and surface contamination?

Intervention outcomes must also distinguish environmental contamination from opportunities for human exposure. An ecological analysis of market closures during the first H7N9 epidemic in China estimated reductions of 97–99% in daily human infections across four cities [30]. Such findings establish the potential importance of interrupting market-associated contact, but do not identify which components could achieve protection while trading continues. Nor does reduced contact necessarily imply elimination of environmental viral material. Stakeholder research in Viet Nam found that trading could shift outside formal markets during temporary closures, emphasizing the relevance of value chains, communication, and implementation beyond the market boundary [31]. Studies of attitudes toward central slaughtering likewise demonstrate that changes to market organization require consideration of stakeholder acceptance [32]. Measures should therefore be evaluated against their intended outcomes: poultry management against circulation and amplification, hygiene against contamination, and personal protection against worker exposure. Persistent environmental detection alone cannot establish that a worker-protection measure has failed.

The prevention rationale in Cambodia is supported by evidence extending beyond environmental viral RNA detection. Previous market-worker surveillance documented serological evidence of subclinical H5N1 and H9N2 infections, while viable viruses of both subtypes were recovered from personal air samplers worn during routine market work [5, 6]. The investigation of 16 human H5N1 infections during February 2023–August 2024 reported sick or dead poultry exposure in every patient and identified genetically similar viruses in poultry associated with case investigations or longitudinal market surveillance [33]. These findings support prevention across poultry–human

interfaces, but do not establish that those human cases originated in markets or identify their transmission routes. Similarly, viral RNA detection on surfaces identifies contamination rather than proving surface-mediated infection. The present study did not measure viability, inhaled dose, or individual exposure, and the environmental findings cannot be translated directly into a reduction in infection risk.

Worker feedback also identified substantial usability barriers alongside mixed willingness to adopt the lid. All five workers answered “No” to the direct question about wanting to use it, yet three agreed with the adapted usability statement that they would like to use it frequently, and three would continue using it if it became a permanent part of the machine. Four reported confidence in using the lid, despite all five considering it too complicated. These responses indicate that confidence, perceived protection, ease of use, and willingness to adopt the device were not interchangeable as aspects of acceptability. The assessment provides formative feedback from early experience with the tested configuration, rather than evidence of sustained adoption. Reported slower work reflects workers’ perceptions, not a measured reduction in processing speed or income. Comparable tensions arise with other interventions: among 216 market workers in Chongqing, only 26.8% supported an overnight-storage ban and 32.4% supported regular rest days [33]. Therefore, evidence of risk reduction cannot substitute for local assessment of operational suitability.

Previous research on knowledge, attitudes, and practices (KAP) helps place market worker responses in context. Studies of poultry vendors have examined how risk perceptions and perceived barriers relate to preventive behavior [34]. In Cambodia, a survey of 100 workers at three markets (including Orussey) retrospectively identified reductions in mask use from 58% to 40% and influenza-related training participation from 30% to 10% during the COVID-19 pandemic, alongside economic pressures [35]. The cross-sectional, retrospective study design limits causal interpretation, but reinforces that preventive practices depend on the conditions under which people work. Earlier Cambodian community studies similarly found that improved knowledge could coexist with persistent risky poultry handling [36]. A behavioral intervention among backyard poultry raisers in Bangladesh increased awareness and expressed willingness without

corresponding changes in observed behavior during follow-up [37]. Although these community findings are indirect evidence for market workers, they caution against equating knowledge, willingness, and sustained adoption. Education and communication should support practical changes, but cannot resolve equipment, infrastructure, workload, or livelihood constraints by themselves.

Future evaluations should consequently connect environmental performance with implementation from the outset. Worker participation should guide redesign, integration with locally manufactured machines, cleaning requirements, and maintenance, followed by longer assessment during routine and high-demand periods. Complementary measures addressing surface hygiene, water and waste management, and poultry holding should be evaluated both separately and in feasible combinations so their additional contributions can be identified. Concurrent poultry, air, and paired surface measurements would help interpret changes due to intervention against the circulating viral background, while more independent sampling dates would strengthen comparisons across activity and environmental conditions. Environmental surveillance can support this iterative evaluation, but surveillance itself does not demonstrate that a mitigation measure works [3, 13, 14]. Costs, responsibilities, workload, and effects on livelihoods should be assessed alongside virological outcomes rather than deferred until a technically promising intervention has already been selected.

Several limitations constrain the conclusions drawn from this study. Intervention allocation was nonrandomized and occurred at the sampling-date level, leaving three independent dates with intervention and three without; samples collected within a date were treated as subsamples rather than independent intervention replicates. Poultry composition and activity levels varied, locally manufactured machines differed, and the numbers of defeathering cycles and duration of active operation during the study period were unavailable. With a small number of independent sampling dates, nonsignificant comparisons cannot establish equivalence or exclude a meaningful effect. The direct comparisons of pre-to-post surface community changes were exploratory and were limited by three independent sampling dates per condition. Multivariate dispersion was not evaluated. Target-enriched metagenomics describes detectable taxa and relative signals rather

than absolute viral concentrations, while eligibility restrictions and limited matched observations constrain interpretation of qPCR comparisons. The five participating operators provided valuable design feedback but do not represent acceptability across the wider workforce.

This study identifies both a potential benefit and clear limits of the tested intervention. Lower airborne viral richness warrants further evaluation, while continuing surface contamination and operational difficulties show why this finding alone is insufficient to support routine adoption. Progress requires establishing which measures produce sustained improvements, which pathways remain unresolved, and whether workers can maintain those measures in everyday practice. Partial lids may contribute to that effort, but their role must be demonstrated within a broader approach that addresses circulating infection, environmental contamination, and worker protection while preserving the functions that make markets essential.

METHODS

Study design and intervention

A nonrandomized field intervention study was conducted in the slaughter area of Orussey Market, Phnom Penh, Cambodia, on six dates: September 16–17 and October 6–9, 2025. Partial lids were installed on September 16 and October 6 and 8; machines operated without lids on September 17 and October 7 and 9. Lid and no-lid dates alternated, with each lid date immediately preceding a no-lid date. Sampling included air, poultry, and plastic sheets under both groups. Dates were arranged with market authorities and workers to accommodate routine operations.

Stalls 1 and 2 were combined as one analytical unit (S1/S2) because of their proximity; stalls 3 and 4 formed separate units (S3 and S4). All participating machines were operated under the same lid condition on each sampling date. The sampling day was therefore the intervention unit, with three independent days per group. Samples from separate analytical units on the same day were treated as within-day subsamples.

Stainless-steel partial lids were custom-fabricated for the locally-manufactured machines, whose upper openings differed in size. Each lid retained a central aperture of 32 cm in diameter, based

559 on a previously evaluated design [11]. The partial-lid configuration is shown in Supplementary Fig.
560 S12C–D. Workers did not remove or displace the lids during the three-hour air-sampling periods.

561 **Environmental and poultry sampling**

562 Air samples were collected using three AerosolSense™ Samplers (Thermo Fisher Scientific,
563 Waltham, MA, USA), with one positioned above the defeathering area at S1/S2, S3 and S4 Each
564 sampler operated throughout a three-hour air-sampling period on each of the six sampling dates,
565 drawing air through an omnidirectional inlet at a nominal flow rate of 200 L/min. A new single-use
566 cartridge was used for each air-sampling period, yielding 18 individual air samples (nine collected
567 on dates with lids and nine on dates without lids, across three dates per group). The collection
568 matrix from each cartridge was rinsed and vortexed in 2 mL of viral transport medium (VTM),
569 following the procedure described previously [13].

570 Sampler positions and orientations were maintained within each analytical unit across all six
571 sampling dates, including dates with and without lids, although their placement differed among
572 units according to stall configuration. Approximate distances from the upper opening of the
573 defeathering machine to the base of the sampler were 90 cm in S1/S2, 62 cm in S3 and 100 cm
574 in S4. Measurements of sampler height above the floor were not available. The market layout and
575 approximate sampler measurements are illustrated in Supplementary Fig. S12A–B, and a
576 representative sampler position is shown in Supplementary Fig. S12E.

577 Three A4-sized plastic sheets (210 × 297 mm) were placed in each of the four participating stalls:
578 one above the defeathering machine and two on nearby walls (Supplementary Fig. S12A, C and
579 E). The entire surface of each sheet was dry swabbed immediately before and after the three-hour
580 air-sampling period (Supplementary Fig. S12F). For downstream analyses, surface samples were
581 grouped by sampling date, analytical unit, and collection time point (before or after the air-sampling
582 period), resulting in 18 pools of samples collected before the air-sampling period and 18 pools of
583 samples collected afterward. Each pool contained six swabs for S1/S2 or three swabs for S3 and
584 S4. Each pair comprised samples collected before and after the air-sampling period from
585 corresponding numbered sheets within the same analytical unit and sampling date. The sheets

remained exposed to routine market activities throughout the air-sampling period, rather than to defeathering alone.

During each three-hour air-sampling period, observers manually recorded the numbers of chickens and ducks processed at each participating stall using field monitoring sheets. Processing events may have been missed if an observer was temporarily away from a stall; the recorded counts may therefore underestimate the total number of birds processed during the air-sampling period. Recorded processing counts by stall and sampling date are provided in Supplementary Table S3 and are distinct from the numbers of birds sampled for virological analyses.

At each participating stall, poultry were sampled from the batch present at the time of the sampling visit. Oropharyngeal and cloacal swabs were collected from 381 birds (231 chickens and 150 ducks), yielding 762 swabs. For pooled analyses, equal volumes of the original oropharyngeal and cloacal swab suspensions were combined by sampling date and analytical sampling unit, regardless of bird species or swab type. This resulted in 18 poultry pools, each representing 10–40 birds (20–80 swabs). Sixteen pools contained samples from both chickens and ducks, while two contained chicken samples only (S3 on 17 September and 6 October). The numbers and species of sampled birds by date and analytical unit are presented in Supplementary Table S1.

The number of defeathering cycles and the exact duration of active machine operation during each air-sampling period were not available. Carcass wash water was used in the defeathering machines, and no clean water was added during the air-sampling period. No cleaning was observed during the three-hour air-sampling periods; routine cleaning took place at the end of the working day.

Overall, 924 specimens were collected: 762 poultry swabs, 144 plastic-sheet swabs, and 18 individual air samples. For downstream analyses, these corresponded to 18 poultry pools, 36 plastic-sheet pools (18 pre air sampling and 18 post air sampling) and 18 individual air samples, yielding 72 analytical sample identifiers. Poultry and surface specimens were collected using polyester swabs and placed in in-house VTM prepared as described by Cronin et al. [13] . All samples were transported on ice to the Institut Pasteur du Cambodge, aliquoted upon arrival, and

stored at −80 °C until processing. RNA extraction and pooling procedures are detailed in the RNA extraction subsection.

Ambient temperature, relative humidity, and local air velocity were recorded at approximately 30-min intervals in each of the four participating stalls using a MASTECH MS6300 environmental meter. Individual readings by sampling date, stall, and recorded time are provided in Supplementary Table S5. Available readings from the four stalls were summarized by sampling date using medians and minimum–maximum ranges. Recorded zeros were retained, and missing or cancelled readings were not imputed. These measurements provided descriptive context for the air-sampling periods and were not included as covariates in the intervention comparisons.

Questionnaire

Worker acceptability and usability were assessed using structured questionnaires administered individually and privately in Khmer using KoboToolbox by a study interviewer. The same five workers completed the pre-use and post-use assessments, which were linked using participant identifiers and de-identified for analysis. The pre-use questionnaire was administered before the initial lid evaluation and assessed perceived occupational risks, previous information or training on illness prevention, routine protective practices, existing machine covers or barriers, cleaning practices and methods, water sources, willingness to try the lid, anticipated difficulties and suggested improvements. The pre-use questionnaire asked whether workers cleaned the defeathering machine “after each use”, but did not specify whether “use” meant one defeathering cycle or an entire working day.

The KoboToolbox records date the post-use interviews to 17 September 2025, following the initial monitored partial-lid session and before the October sampling dates. The post-use questionnaire assessed perceived protection, satisfaction, comfort, perceived effects on workflow, willingness to use the lid in its tested form or as a permanent machine component, and suggested modifications; it also included ten items adapted from the SUS. Questions about barriers allowed multiple responses. The same five workers operated their respective machines during all three air-sampling periods with partial lids. Questionnaire responses were summarized descriptively as counts out of five workers, and adapted SUS items were reported individually rather than combined into a

standard SUS score. This formative assessment reflected perceptions following the initial monitored partial-lid session, not sustained use across a full working shift; reports of slower work were not direct measurements of processing time. Full questionnaires are provided in Supplementary Material 2.

Ethics

All research procedures were conducted in accordance with relevant ethical guidelines and national regulations. The study protocol, including sample collection and the administration of pre- and post-intervention questionnaires to market workers, was approved by the National Ethics Committee for Health Research (NECHR), Ministry of Health, Cambodia (approval no. NECHR357, 2025). Written informed consent was obtained from all five participating workers. Poultry swab collection was conducted in collaboration with the National Animal Health and Production Institute and with authorization from the General Directorate of Animal Health and Production, Ministry of Agriculture, Forestry and Fisheries, Cambodia.

RNA extraction

RNA extraction for AIV and NDV RT-qPCR and metagenomic sequencing was performed using the Direct-zol RNA Miniprep Kit (Zymo Research, California, USA), following the procedure described by Cronin et al. [13] and the manufacturer's instructions. Individual specimens or prepared pooled suspensions were vortexed for 15–30 s before extraction. For each extraction, 200 µL of sample was processed, and RNA was eluted in 50 µL of nuclease-free water.

Two sample-processing approaches were used. For individual AIV and NDV RT-qPCR testing, RNA was extracted separately from individual poultry and plastic-sheet swab specimens. For pooled RT-qPCR and metagenomic analyses, equal volumes of the original swab suspensions were combined before RNA extraction according to sampling date and analytical sampling unit. Poultry pools combined oropharyngeal and cloacal swab specimens without separation by bird species or swab type. Plastic-sheet specimens collected during the pre-sampling and post-sampling periods were pooled separately. RNA extracted from the resulting pools was used for

pool-level AIV and NDV RT-qPCR and metagenomic sequencing. Air samples were processed individually without pooling.

IBV testing was performed after the original RNA extracts had been discarded. Therefore, individual specimens retained in viral transport medium (VTM) were retrieved, and RNA was re-extracted separately using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Briefly, 140 µL of each specimen was mixed with 560 µL of Buffer AVL containing carrier RNA and incubated at room temperature for 10 min. Following ethanol addition and column centrifugation, the RNA was washed with Buffers AW1 and AW2 and eluted in 60 µL of Buffer AVE. No pooling was performed for IBV RT-qPCR.

AIV and NDV RT-qPCR and metagenomic sequencing used Direct-zol RNA extracts obtained from individual or pooled specimens, as appropriate, whereas IBV RT-qPCR used independently extracted individual specimens processed with QIAamp Viral RNA Mini Kit. The extraction protocol for each viral target was consistent across sampling dates, with and without lids. Consequently, comparisons between IBV RT-qPCR and metagenomic measurements involved RNA obtained from separate extractions.

Targeted RT-qPCR

RT-qPCR targeted the influenza A matrix (M) gene using the approach reported by Siegers et al. [38], with reverse transcription at 50 °C for 30 min, incubation at 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 55 °C for 30 s. NDV testing used the M-gene primer–probe assay described by Wise et al. [39], with modified cycling conditions: 50 °C for 15 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. IBV testing used the 5′ untranslated region primer–probe assay described by Callison et al. [40], with adapted conditions: 50 °C for 15 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 58 °C for 60 s.

Reactions were performed using the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen, USA) on a CFX real-time PCR system (Bio-Rad, USA), under assay-specific cycling conditions. Each run included positive, no-template, and extraction-negative controls.

Cycle-threshold (Ct) values and laboratory result classifications were obtained from the original laboratory worksheets. Only numeric Ct values ≤40 that were not flagged as unsuitable for interpretation were included in numerical comparisons and Ct–RPM correlations. Values >40, no detects, and results flagged as unsuitable for interpretation were excluded from numerical analyses without assigning arbitrary Ct values.

Library preparation and next-generation sequencing

Library preparation and sequencing followed Cronin et al. [13], with minor modifications. Extracted RNA was converted to cDNA using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) with random primers, followed by the NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Kit. Illumina-compatible libraries were generated using the Twist Library Preparation Enzymatic Fragmentation Kit 2.0 (Twist Bioscience, San Francisco, CA, USA) and enriched using the Twist Comprehensive Viral Research Panel according to the manufacturers' protocols. Enriched libraries were pooled at equimolar concentrations, denatured, and diluted following the MiSeq System Denature and Dilute Libraries Guide, then sequenced on an Illumina MiSeq using a MiSeq v3 reagent kit to generate paired-end 75-bp reads. Nuclease-free water served as a negative control from extraction through sequencing. Negative controls contained few detectable viral contigs, none of which were shared with study samples (Supplementary Fig. S13). These findings provided no evidence of shared viral-contig contamination in the sequenced controls, although they cannot exclude all forms of contamination.

Processing of viral sequencing data and abundance estimation

Raw reads were processed using the SeqtoID metagenomics pipeline (formerly CZ ID; https://czid.org/), as previously described [41]. Counts of reads mapping directly to de novo assembled viral contigs were downloaded from the pipeline. Taxonomic filtering excluded contigs assigned to viruses of fungi, plants, or bacteria, including Caudoviricetes and Crassvirales. In this study, a viral taxon refers to a distinct taxonomic assignment represented as a feature in the filtered viral read-count table. These assignments may be at species level or a higher taxonomic rank. Reads per million (RPM) were calculated as the reads assigned to each viral taxon divided by the

total viral reads in the corresponding library, multiplied by 10^6. RPM therefore represented relative viral-read abundance rather than an absolute concentration. The filtering procedure counted reads mapping to viral contigs only if they comprised more than 0.1% of total reads in at least one sample; counts failing this criterion were set to zero.

Virus diversity estimation

Viral read counts were used to calculate Bray–Curtis and Jaccard dissimilarities with vegdist in vegan [42]. Principal coordinate analysis (PCoA) visualized community composition. Pairwise PERMANOVA used adonis2 on Bray–Curtis distances calculated from raw counts for 116 avian-list taxon features. Groups sampled together were compared by exact swaps within matched sampling-day and analytical-unit pairs. Poultry comparisons retained the nine pools from the three dates shared with the comparator group. Comparisons between lid groups used whole-day permutations of six sampling-day blocks, with three days per group and 19 non-identity permutations. Benjamini–Hochberg adjustment was applied across the 21 pairwise comparisons. Unrestricted sample-level permutation results were retained separately for reference and were not used for the reported pairwise inference (Supplementary Data 1). Observed richness, Chao1, Shannon, and Simpson indices were generated with estimate_richness in phyloseq v1.54.2. Observed richness was calculated as the number of taxonomic features with a non-zero read count in each sample after filtering. The other alpha-diversity metrics characterized within-sample diversity.

Leave-one-taxon-out analyses characterized the contributions of 73 poultry-associated taxa to variation among air samples and among plastic-sheet samples separately. Bray–Curtis distances used raw read counts, and Jaccard distances used presence–absence data, calculated with vegdist in vegan. Each taxon was removed in turn and distances recalculated for all 153 pairs among 18 air samples and all 630 pairs among 36 surface samples. Signed change was defined as the distance with the taxon minus the distance without it. Positive values indicated increased dissimilarity and negative values indicated increased similarity. Mean absolute changes ranked taxa, while mean signed changes described their overall direction of contribution. Contributions were nonadditive and did not represent proportions of variance explained. PCA of taxon-specific

distance changes used prcomp for visualization. These analyses were descriptive and did not isolate contrasts between lid groups or sampling periods (Supplementary Data 2).

Statistical analysis and graphical representation

Metagenomic analyzes used R v4.5.2, [43] and RT-qPCR Ct comparisons and Ct–RPM correlations used GraphPad Prism v11.1.0 (GraphPad Software, Boston, MA, USA). Alpha-diversity comparisons across more than two groups used Kruskal–Wallis tests followed by Dunn's post hoc tests, implemented with kwAllPairsDunnTest in PMCMRplus v1.9.12. Two-group comparisons used wilcox.test in base R. A sensitivity analysis of individual air-sample taxon counts used a linear mixed-effects model fitted with lmer in lmerTest v3.2.1, with lid use and stall unit as fixed effects and sampling day as a random intercept. The estimated lid effect was reported as a count difference with a 95% confidence interval and a P value based on Satterthwaite degrees of freedom. Metagenomic P values were corrected for false discovery rate using the Benjamini–Hochberg method. Significance annotations were *P < 0.05, **P < 0.01, and ***P < 0.001 unless otherwise stated. Most plots used ggplot2 v4.0.3 [44], and heatmaps used Heatmap in ComplexHeatmap v2.26.1. For each virus, RPM values were ranked across samples, ties received average ranks, and ranks were scaled from zero to one by subtracting the minimum rank and dividing by the difference between maximum and minimum ranks. Viruses with identical values across all samples received scaled values of zero. These heatmaps show within-virus variation across samples rather than abundance differences between viruses.

The primary RT-qPCR intervention comparisons used daily median Ct values calculated from eligible numeric results in S1/S2, S3, and S4. Exact two-sided Mann–Whitney U tests compared three days with partial lids and three days without lids. Secondary sample-level comparisons used the same test without adjustment for within-day clustering. Numerical analyses included only Ct values meeting the eligibility criteria described under Targeted RT-qPCR. Frequencies of laboratory result categories were summarized descriptively. Day-level observed-richness comparisons also used exact two-sided tests.

A daily median RPM value was calculated from the three individual air samples on each sampling date, separately for *Alphainfluenzavirus influenzae* (AIV), *Orthoavulavirus javaense* (NDV), and

the broader avian coronavirus assignment. Daily medians were then compared between days with partial lids and days without lids (three days per group) using exact two-sided Mann–Whitney U tests. Associations between RT-qPCR Ct values and virus abundance were assessed using two-sided Spearman rank correlations, separately by sample type, across both lid groups. Only matched observations with valid numeric Ct values ≤40 were included, while zero RPM values were retained. Correlations were not estimated when either variable lacked variation. Ct–RPM correlations were evaluated for AIV, NDV, and IBV in air samples, and for AIV and NDV in pooled poultry and plastic-sheet samples. The Ct–RPM correlations assessed associations between RT-qPCR and metagenomic measurements across both lid groups. Differences between lid groups were evaluated separately using the day-level Ct and RPM comparisons described above. For graphical presentation, RPM values were displayed as $\log_{10}(\text{RPM} + 1)$.

For molecular analyses, Benjamini–Hochberg correction was applied within five separate families of tests. The first three families comprised three day-level air Ct comparisons, three day-level air RPM comparisons, and three sample-level air Ct comparisons, respectively. The remaining two families comprised the three air Ct–RPM correlations and five Ct–RPM correlations in poultry and plastic-sheet samples. One AIV correlation for plastic-sheet samples collected before the air-sampling period could not be estimated and was excluded. To describe temporal variation in the sampled poultry, AIV and NDV RT-qPCR results were summarized by sampling date as numeric Ct values, nondetects, and poor-quality amplification results. Daily median Ct values were calculated from valid numeric results only, without formal comparisons between dates.

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Author contributions

F.E.S., P.C., V. Heang, S.T., S. Sin, J.S., G.J.D.S., C.F., P.F.H., and E.A.K. contributed to the conceptualization of the study. F.E.S., P.C., V. Heang, C.F., P.F.H., and E.A.K. were responsible for data curation. Formal analyses were performed by F.E.S., P.C., and V. Heang. Funding was acquired by E.A.K. Investigation and resources were provided by F.E.S., P.C., V. Heang, S.T., S. Sin, C.R., L.K., S.N., C.T., L.L., S. Saulim, L.H., B.B., K.S., V. Hul, K.C., V.S.H., S.R., J.S., G.J.D.S., C.F., P.F.H., and E.A.K. Methodology was developed by F.E.S., P.C., V. Heang, S.T., S. Sin, J.S., G.J.D.S., C.F., P.F.H., and E.A.K. Project administration was undertaken by F.E.S., P.C., G.J.D.S., C.F., P.F.H., and E.A.K. Software was provided by F.E.S., P.C., and V. Heang. G.J.D.S., C.F., P.F.H., and E.A.K. supervised and validated the work. Visualizations were prepared by F.E.S. and P.C. The original draft was written by F.E.S., P.C., V. Heang, and C.R. Review and editing of the manuscript were performed by F.E.S., P.C., V. Heang, G.J.D.S., C.F., P.F.H., and E.A.K. All authors reviewed and approved the final manuscript.

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Data availability

Study sequence data: European Nucleotide Archive accession PRJEB129242.

Code availability

Analysis scripts, processed data tables and figure-generation code: Zenodo [10.5281/zenodo.23793089] and GitHub [https://github.com/yvonnesuLoVE/LBM-Lid-Intervention].

Supplementary information

Supplementary Material 1 contains Tables S1–S7. Supplementary Material 2 contains the pre- and post-intervention questionnaires, including the adapted 10-item usability section. Supplementary figures comprise day-level air RT-qPCR comparisons (S1); additional alpha-diversity metrics (S2); Jaccard ordination (S3); taxon contributions to dissimilarity among air samples (S4); Ct–RPM associations in pooled poultry and plastic-sheet samples (S5); observed viral richness in plastic-sheet pools (S6); the magnitude of before-to-after changes in surface community composition, measured using Bray–Curtis dissimilarity (S7); surface community trajectories and the direct multivariate comparison of before-to-after changes between lid conditions (S8); taxon abundances by lid condition before and after sampling (S9); taxa showing positive estimated before-to-after changes on no-lid days but not on lid days (S10); taxon contributions to dissimilarity among plastic-sheet samples (S11); market layout, partial-lid intervention, air-sampler positioning, and plastic-sheet surface-sampling setup (S12); and viral profiles in negative controls and study samples (S13). Supplementary Data 1 provides pairwise PERMANOVA outputs, and Supplementary Data 2 provides descriptive leave-one-taxon-out contribution outputs for air and plastic-sheet samples.

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1. Viral composition across sample types and sampling dates.

(a) Relative-abundance profiles of poultry-associated viral taxa in poultry and air samples, grouped by sampling date and analytical sampling unit (S1/S2, S3, and S4). Each stacked bar represents one analytical sample. Colors identify the viral taxa shown in the legend, with additional taxa grouped as “Others.” Air samples are labeled according to lid use. (b) Heatmap of rank-scaled reads per million (RPM) for viral taxa detected across poultry, air, and plastic-sheet samples. Rows represent viral taxa and columns represent analytical samples, arranged by sampling date and sample type. For each virus, RPM values were ranked across samples and rescaled to the interval 0–1, with increasing color intensity indicating a higher within-virus rank. The annotation above the heatmap identifies poultry samples in green, air samples in blue, plastic-sheet samples collected before the air-sampling period in yellow, and those collected afterward in purple. Plastic sheet samples were collected before and after the three-hour air-sampling period. Samples were collected on six dates in September and October 2025.

Figure 2. Viral alpha diversity and observed richness across sample types and lid use

(a) Simpson diversity indices for poultry, air, and plastic sheet samples. Air samples are distinguished by lid use, while plastic-sheet samples are additionally separated by collection before or after the three-hour air-sampling period. Boxplots summarize sample-level distributions, with individual observations overlaid as points. Brackets identify the pairwise comparisons displayed in the panel. Alpha-diversity comparisons across sample groups were performed using the Kruskal–Wallis test followed by Dunn’s post hoc test, as described in Methods. (b) Observed viral richness, expressed as the number of detected viral taxa, in poultry and air samples from S1/S2, S3, and S4 on each sampling date. Green bars represent poultry samples, blue bars represent air samples collected with partial lids, and red bars represent air samples collected without lids. (c) Paired differences in Simpson diversity, calculated as the poultry value minus the air value for samples matched by sampling date and analytical unit. Violin plots and embedded

boxplots summarize the distributions under partial-lid and no-lid use; points represent individual matched pairs. Each group comprised nine unit-day pairs collected across three sampling dates. The exploratory sample-level comparison used a Wilcoxon test (P = 0.077) without adjustment for within-day clustering.

Figure 3. Viral community composition and taxon-specific relative abundance across sample types and lid use.

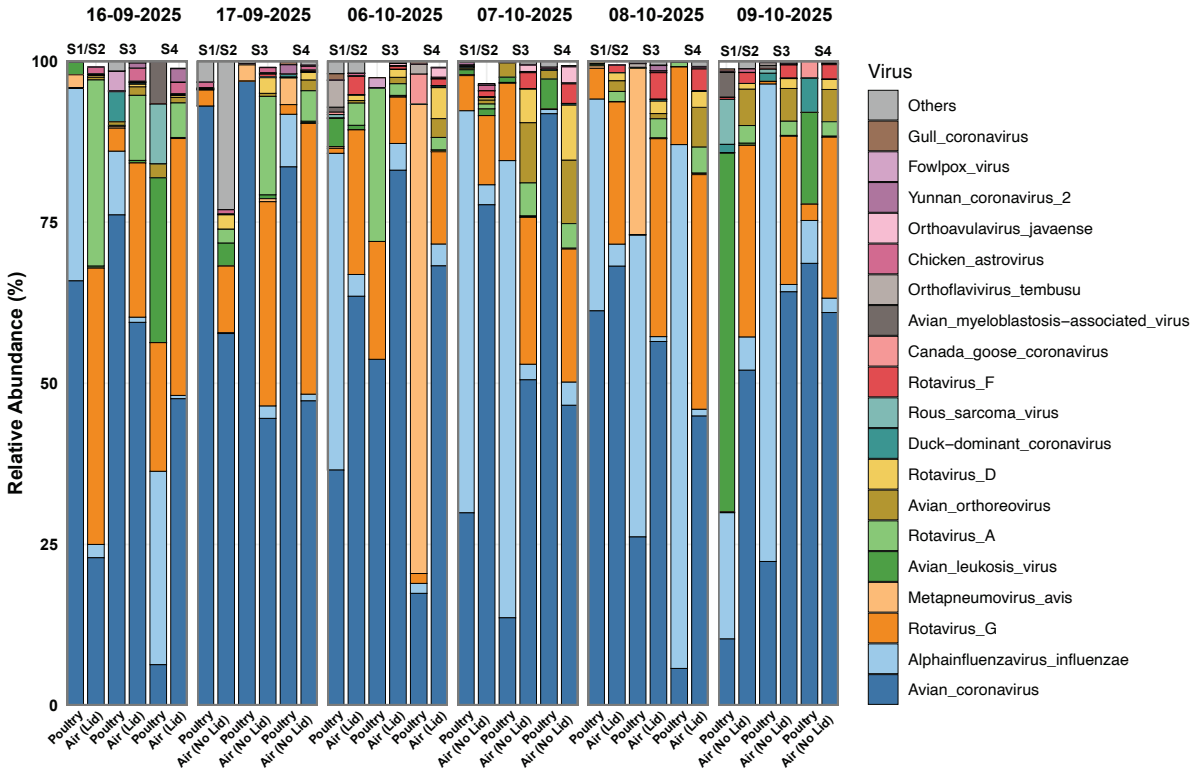
(a) Principal coordinate analysis (PCoA) of Bray–Curtis dissimilarity among poultry, air, and plastic-sheet samples. Each point represents one analytical sample. Colors distinguish sample types and lid use, with plastic-sheet samples further separated by collection before or after the three-hour air-sampling period. Ellipses outline the plotted sample groups. The first two principal coordinates explain 31.49% and 20.06% of the variation, respectively. The overall PERMANOVA result displayed above the ordination refers to the comparison across sample groups (R^2 = 0.40, P = 0.001). (b) Bray–Curtis distance-to-centroid summaries using the poultry sample group as the reference. Boxplots show the distributions for each sample group, with individual observations overlaid. Brackets indicate the displayed comparisons between poultry and air samples collected without lids (P = 0.030), and between the two air groups (P = 0.070). (c) Heatmap showing the direction of taxon-specific relative enrichment for three pairwise comparisons: poultry versus air without lids, poultry versus air with lids, and air without lids versus air with lids. Columns represent viral taxa and rows represent comparisons. Colors identify the group with higher relative abundance: green for poultry, red for air without lids, and blue for air with lids. Asterisks identify comparisons marked as statistically significant. Colors indicate direction rather than magnitude of relative enrichment.

Figure 4. Associations between targeted RT-qPCR Ct values and metagenomic relative abundance in matched air samples.

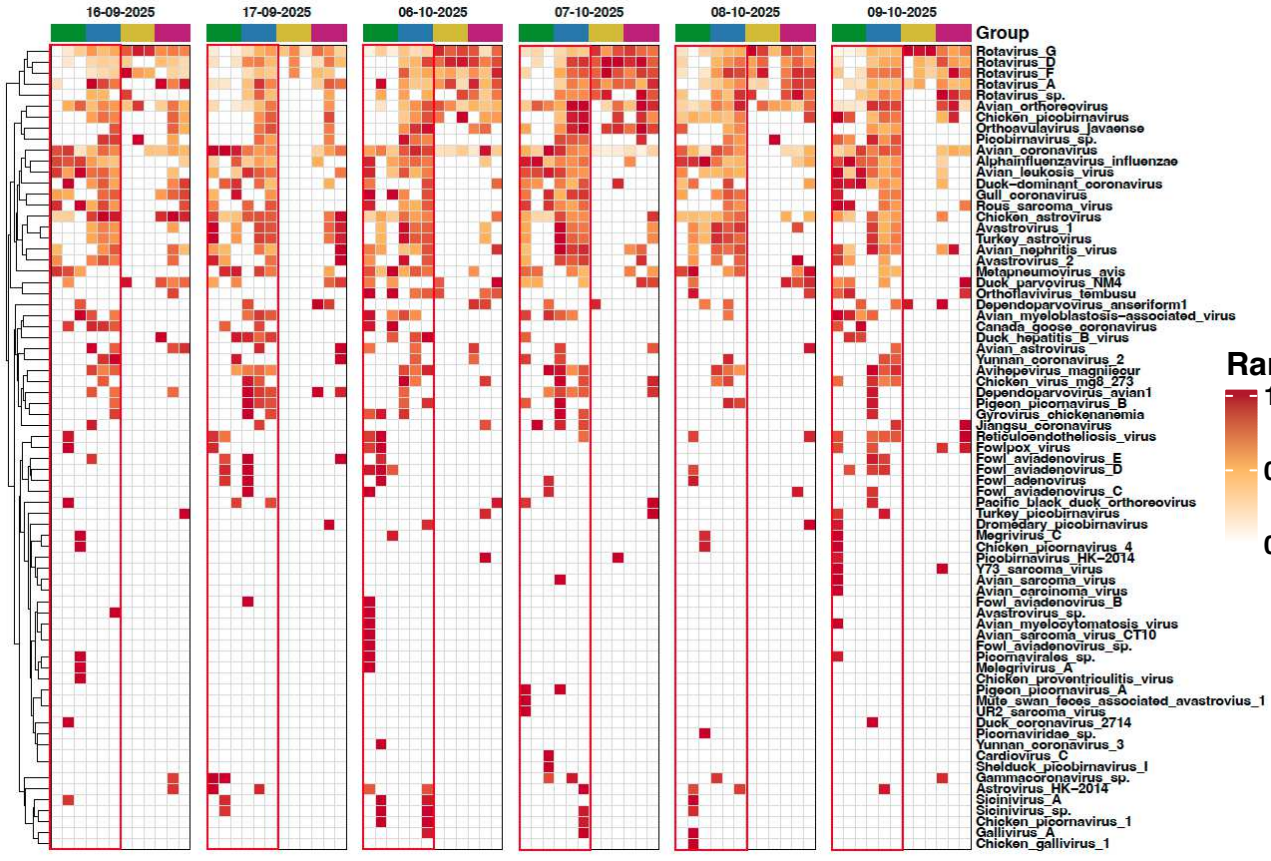
Scatter plots show RT-qPCR cycle-threshold (Ct) values versus $\log_{10}$(RPM + 1), where RPM denotes reads per million, for (a) influenza A virus (AIV; M-gene Ct values versus RPM assigned to *Alphainfluenzavirus influenzae*), (b) Newcastle disease virus (NDV; M-gene Ct values versus RPM assigned to *Orthoavulavirus javaense*), and (c) infectious bronchitis virus (IBV; 5′-UTR Ct

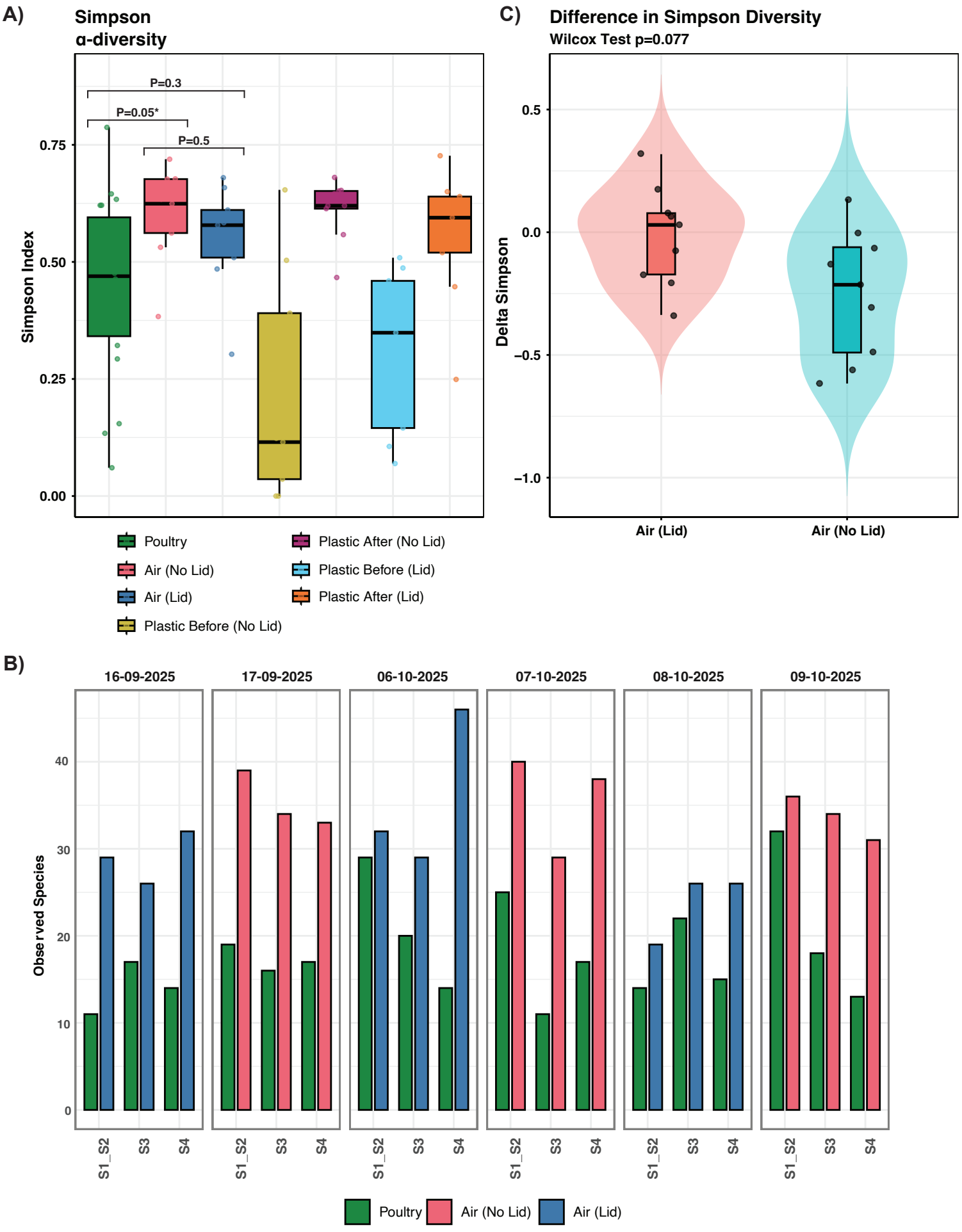
values versus RPM assigned to the broader avian coronavirus taxonomic category). Each point represents one matched AerosolSense air sample; blue and red indicate samples collected with and without partial lids, respectively. Only eligible numeric Ct values ≤40 were included, while zero RPM values were retained. Sample sizes were 17 for AIV (9 with partial lids, 8 without), 18 for NDV (9 per group), and 16 for IBV (9 with partial lids, 7 without), across six sampling dates (three per group). Two-sided Spearman rank correlations were calculated across both lid groups, with Benjamini–Hochberg false-discovery rate correction applied across the three viral targets. Spearman's ρ, 95% confidence intervals, unadjusted P values, and FDR-adjusted q values are shown beneath each panel. The IBV-targeted assay was compared with a broader metagenomic coronavirus assignment, and the corresponding measurements were obtained from separate RNA extractions. Correlation estimates and associated uncertainty measures did not account for within-day clustering. These analyses assessed correspondence between molecular measurements rather than intervention effectiveness.

A)

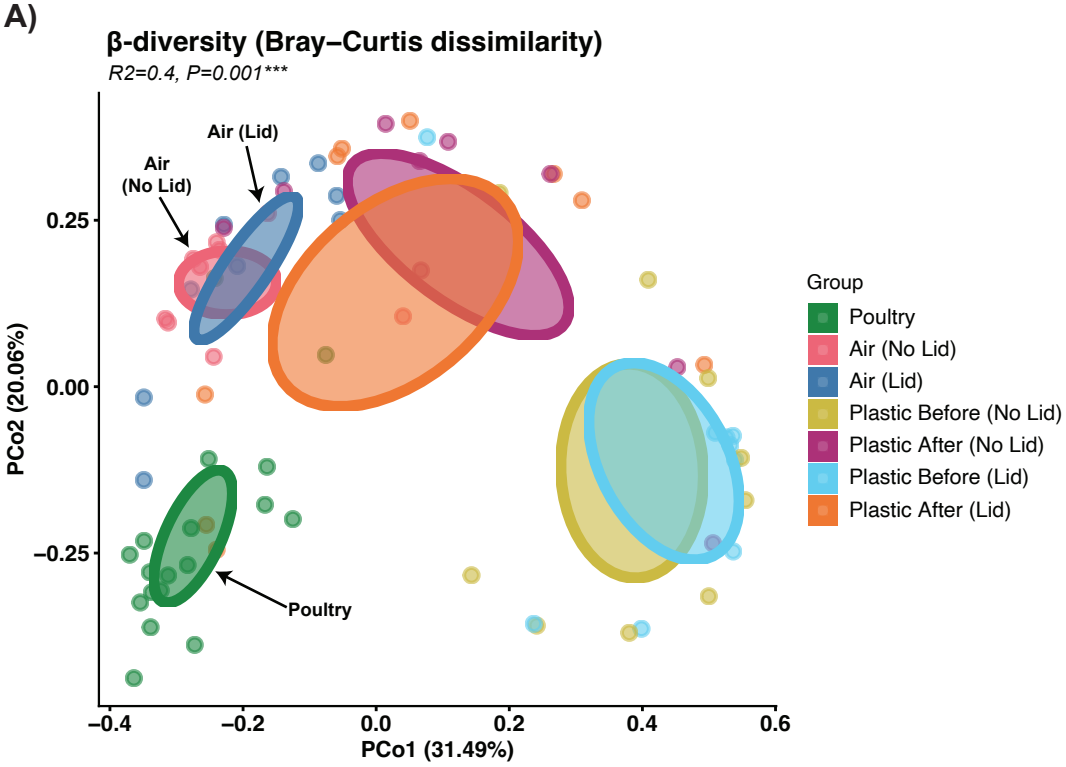

16-09-2025
17-09-2025
06-10-2025
07-10-2025
08-10-2025
09-10-2025
S1/S2
S3
S4
Relative Abundance (%)
100
75
50
25
0
Poultry
Air (Lid)
Air (No Lid)
Virus
Others
Gull_coronavirus
Fowlpox_virus
Yunnan_coronavirus_2
Orthoavulavirus_javaense
Chicken_astrovirus
Orthoflavivirus_tembusu
Avian_myeloblastosis–associated_virus
Canada_goose_coronavirus
Rotavirus_F
Rous_sarcoma_virus
Duck–dominant_coronavirus
Rotavirus_D
Avian_orthoreovirus
Rotavirus_A
Avian_leukosis_virus
Metapneumovirus_avis
Rotavirus_G
Alphainfluenzavirus_influenzae
Avian_coronavirus

B)

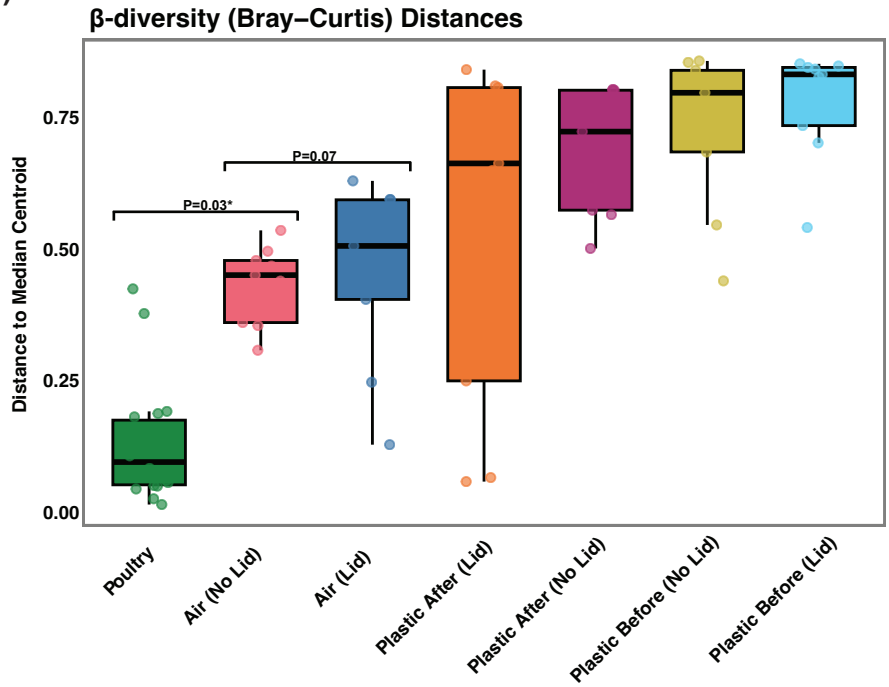

16-09-2025
17-09-2025
06-10-2025
07-10-2025
08-10-2025
09-10-2025
Group
Rotavirus_G
Rotavirus_D
Rotavirus_F
Rotavirus_A
Rotavirus_sp.
Avian_orthoreovirus
Chicken_picobirnavirus
Orthoavulavirus_javaense
Picobirnavirus_sp.
Avian_coronavirus
Alphainfluenzavirus_influenzae
Avian_leukosis_virus
Duck–dominant_coronavirus
Gull_coronavirus
Rous_sarcoma_virus
Chicken_astrovirus
Avastrovirus_1
Turkey_astrovirus
Avian_nephritis_virus
Avastrovirus_2
Metapneumovirus_avis
Duck_parvovirus_NM4
Orthoflavivirus_tembusu
Dependoparvovirus_anseriform1
Avian_myeloblastosis–associated_virus
Canada_goose_coronavirus
Duck_hepatitis_B_virus
Avian_astrovirus
Yunnan_coronavirus_2
Avihepevirus_magniiecur
Chicken_virus_mg8_273
Dependoparvovirus_avian1
Pigeon_picornavirus_B
Gyrovirus_chickenanemia
Jiangsu_coronavirus
Reticuloendotheliosis_virus
Fowlpox_virus
Fowl_aviadenovirus_E
Fowl_aviadenovirus_D
Fowl_adenovirus
Fowl_aviadenovirus_C
Pacific_black_duck_orthoreovirus
Turkey_picobirnavirus
Dromedary_picobirnavirus
Megrivirus_C
Chicken_picornavirus_4
Picobirnavirus_HK–2014
Y73_sarcoma_virus
Avian_sarcoma_virus
Avian_carcinoma_virus
Fowl_aviadenovirus_B
Avastrovirus_sp.
Avian_myelocytomatosis_virus
Avian_sarcoma_virus_CT10
Fowl_aviadenovirus_sp.
Picornavirales_sp.
Melegrivirus_A
Chicken_proventriculitis_virus
Pigeon_picornavirus_A
Mute_swan_feces_associated_avastrovius_1
UR2_sarcoma_virus
Duck_coronavirus_2714
Picornaviridae_sp.
Yunnan_coronavirus_3
Cardiovirus_C
Shelduck_picobirnavirus_I
Gammacoronavirus_sp.
Astrovirus_HK–2014
Sicinivirus_A
Sicinivirus_sp.
Chicken_picornavirus_1
Gallivirus_A
Chicken_gallivirus_1
Rank
1
0.5
0
Group
Poultry
Air
Plastic Before
Plastic After


A)
Simpson
α-diversity
P=0.3
P=0.05*
P=0.5
Simpson Index
0.75
0.50
0.25
0.00
Poultry
Air (No Lid)
Air (Lid)
Plastic Before (No Lid)
Plastic After (No Lid)
Plastic Before (Lid)
Plastic After (Lid)
C)
Difference in Simpson Diversity
Wilcox Test p=0.077
Delta Simpson
0.5
0.0
−0.5
−1.0
Air (Lid)
Air (No Lid)
B)
16-09-2025
17-09-2025
06-10-2025
07-10-2025
08-10-2025
09-10-2025
Observed Species
40
30
20
10
0
S1_S2
S3
S4
Poultry
Air (No Lid)
Air (Lid)

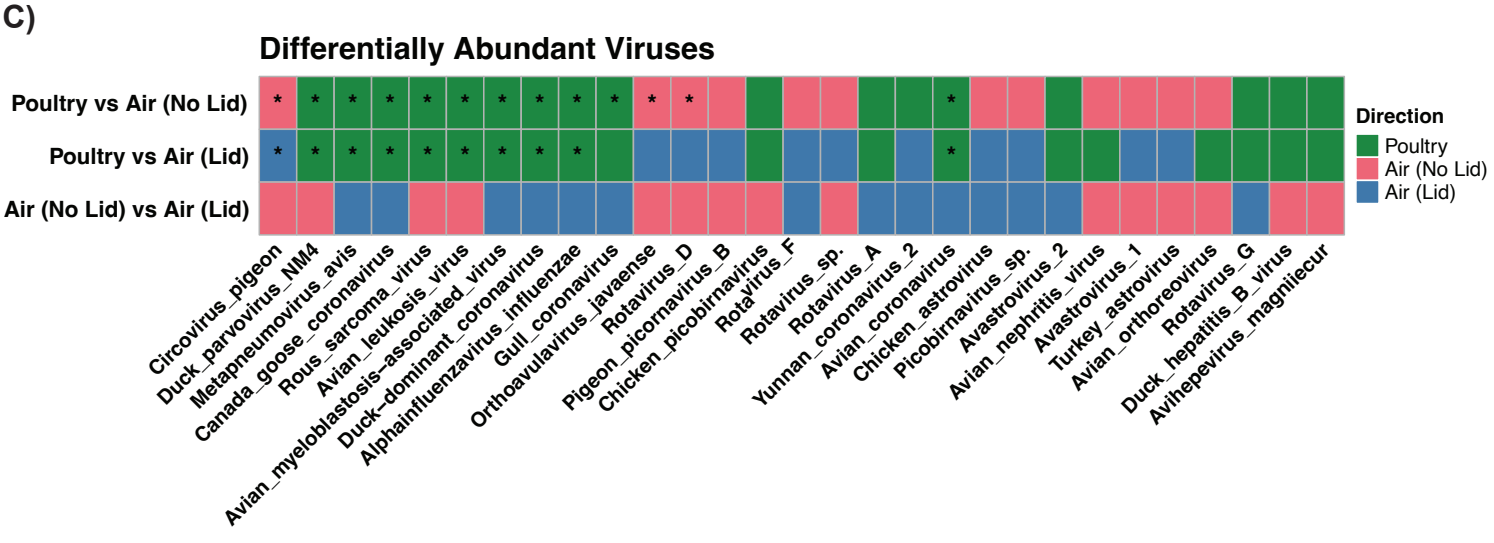
A)

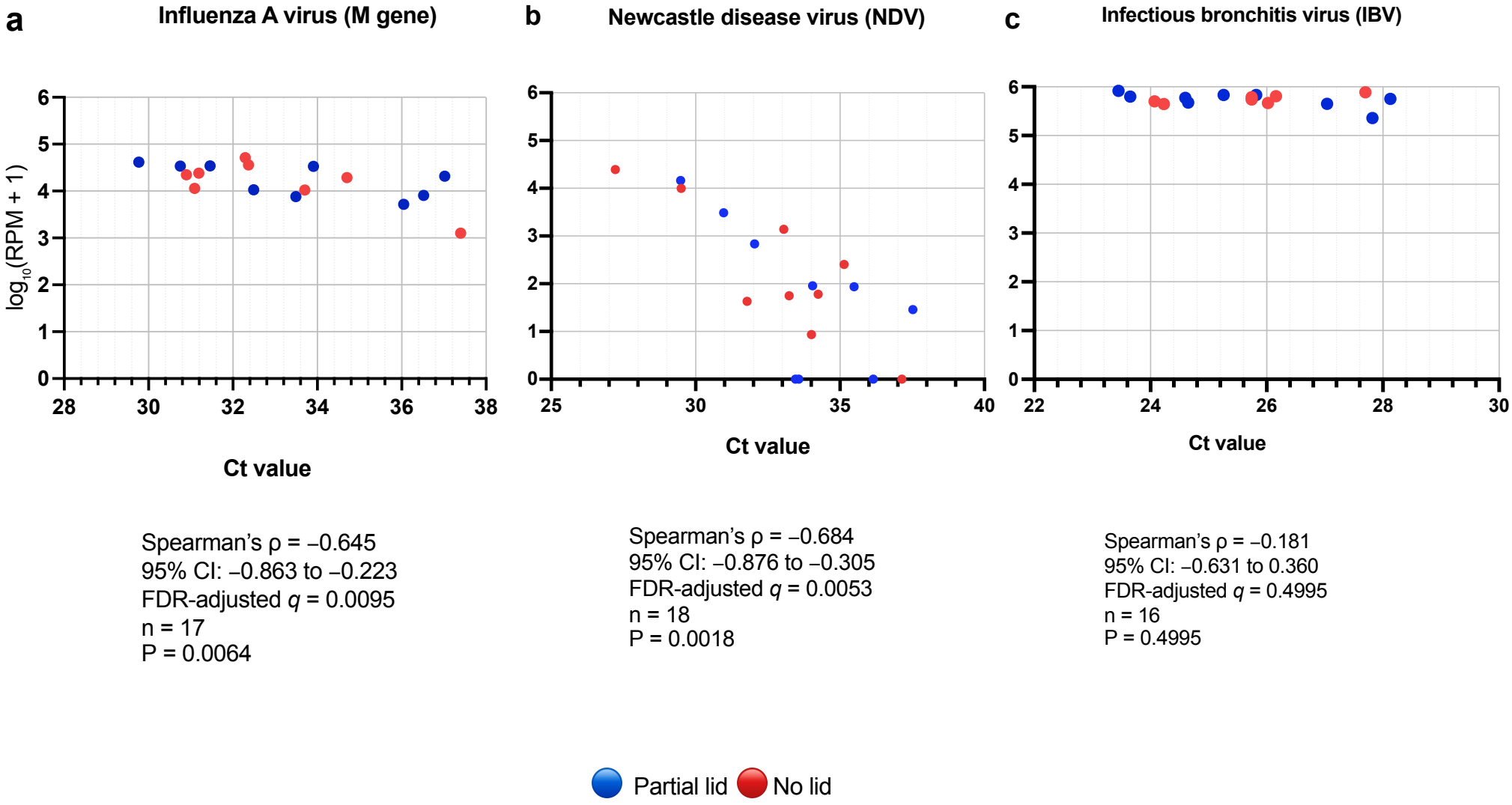

β-diversity (Bray–Curtis dissimilarity)
R2=0.4, P=0.001***
Air (Lid)
Air (No Lid)
Poultry
PCo2 (20.06%)
PCo1 (31.49%)
0.25
0.00
−0.25
−0.4
−0.2
0.0
0.2
0.4
0.6
Group
Poultry
Air (No Lid)
Air (Lid)
Plastic Before (No Lid)
Plastic After (No Lid)
Plastic Before (Lid)
Plastic After (Lid)

B)


β-diversity (Bray–Curtis) Distances
Distance to Median Centroid
0.75
0.50
0.25
0.00
P=0.07
P=0.03*
Poultry
Air (No Lid)
Air (Lid)
Plastic After (Lid)
Plastic After (No Lid)
Plastic Before (No Lid)
Plastic Before (Lid)

C)


Differentially Abundant Viruses
Poultry vs Air (No Lid)
Poultry vs Air (Lid)
Air (No Lid) vs Air (Lid)
Direction
Poultry
Air (No Lid)
Air (Lid)
Circovirus_pigeon
Duck_parvovirus_NM4
Metapneumovirus_avis
Canada_goose_coronavirus
Rous_sarcoma_virus
Avian_leukosis_virus
Avian_myeloblastosis–associated_virus
Duck–dominant_coronavirus
Alphainfluenzavirus_influenzae
Gull_coronavirus
Orthoavulavirus_javaense
Rotavirus_D
Pigeon_picornavirus_B
Chicken_picobirnavirus
RotaVirus_F
Rotavirus_sp.
Rotavirus_A
Rotavirus_2
Yunnan_coronavirus
Avian_coronavirus
Chicken_astrovirus
Picobirnavirus_sp.
Avastrovirus_2
Avian_nephritis_virus
Avastrovirus_1
Turkey_astrovirus
Avian_orthoreovirus
Rotavirus_G
Duck_hepatitis_B_virus
Avihepevirus_magniiecur


a
Influenza A virus (M gene)
b
Newcastle disease virus (NDV)
c
Infectious bronchitis virus (IBV)
log10(RPM + 1)
Ct value
Ct value
Ct value
Spearman's ρ = –0.645
95% CI: –0.863 to –0.223
FDR-adjusted q = 0.0095
n = 17
P = 0.0064
Spearman's ρ = –0.684
95% CI: –0.876 to –0.305
FDR-adjusted q = 0.0053
n = 18
P = 0.0018
Spearman's ρ = –0.181
95% CI: –0.631 to 0.360
FDR-adjusted q = 0.4995
n = 16
P = 0.4995
Partial lid
No lid

Supplementary Files

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- SupplementaryFigureS10A4.pdf
- SupplementaryFigureS6Observedrichness.pdf
- SupplementaryFigureS13A4.pdf
- SupplementaryFigureS2Chao1Shannon.pdf
- SupplementaryFigureS3Jaccard.pdf
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- SupplementaryMaterial2.Questionnaire.pdf
- SupplementaryData2AIRPLASTIC.xlsx
- SupplementaryData1PairwisePERMANOVA.xlsx