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Integrated Culture-Dependent and qPCR Monitoring Reveals Persistent *Penicillium* Contamination Throughout Smear-Ripened Cheese Production[§]

Running head: Integrated Monitoring of *Penicillium* in Cheese

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SUMMARY

Research background. Smear-ripened cheeses rely on surface microbiota dominated by *Brevibacterium*, coryneform bacteria, and yeasts to develop their characteristic sensory properties. However, open-surface production increases the risk of airborne mold contamination, particularly by *Penicillium* spp., which may compromise product quality and shelf life. Identifying contamination sources and critical control points is essential for effective HACCP-based risk management.

Experimental approach. Environmental and product-associated microbiota were monitored in an industrial smear-ripened cheese facility using cultivation, microscopy, and real-time PCR. Environmental contamination was assessed at 24 locations during two sampling periods before and after ethylene oxide fumigation. In parallel, microbiological succession was monitored in two cheese batches throughout ten technological stages of production.

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Results and conclusions. Penicillium spp. represented the predominant contamination risk within the production environment and on cheese surfaces. Real-time PCR detected fungal DNA more frequently than cultivation, demonstrating the value of combining molecular and culture-dependent approaches. Ethylene oxide fumigation reduced fungal contamination associated with cheese production, although environmental recolonization was observed at facility entry points and cold-storage areas. Key contamination hotspots were identified during forming, drying, washing, and packaging operations. The results indicate that integrated environmental and product monitoring can effectively support contamination tracking and targeted hygiene interventions in smear-ripened cheese production.

Novelty and scientific contribution. This study demonstrates the practical value of combining environmental surveillance with product microbiota monitoring to identify contamination sources and verify hygiene measures in industrial cheese production. The integrated use of cultivation and molecular methods provides a robust framework for HACCP verification and contamination management in smear-ripened cheese facilities.

Keywords: smear-ripened cheese; environmental microbiota; *Brevibacterium*; *Penicillium*; real-time PCR; HACCP

INTRODUCTION

Smear-ripened cheeses are characterized by complex surface microbial communities that determine the distinctive sensory properties of the final product. Surface microbiota, typically dominated by *Brevibacterium linens*, coryneform bacteria, and yeasts, contribute to the development of characteristic colour, texture, and flavour through proteolytic and lipolytic activities occurring during ripening [*1,2*].

The production of smear-ripened cheeses involves repeated surface treatment with brine and ripening cultures. Although this process is essential for the development of desirable sensory attributes, it simultaneously increases the susceptibility of cheese surfaces to colonization by environmental microorganisms [*3,4*]. Airborne fungal contamination represents a persistent challenge in dairy production due to its potential impact on product quality, shelf-life, and, in some cases, food safety [*5*]. Species of the genus *Penicillium* are among the most frequently reported fungal contaminants in cheese production environments and are commonly associated with spoilage of smear-ripened cheeses [*6,7*]. Previous studies have identified the production environment as an

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important reservoir and source of microbial contamination, with airborne dissemination representing a major route of transfer between environmental niches and cheese surfaces [*8,9*].

Routine microbiological surveillance in dairy facilities is based primarily on culture-dependent techniques. However, cultivation methods may underestimate microbial diversity because a proportion of microorganisms remains undetectable under standard laboratory conditions [*10,11*]. Therefore, culture-independent molecular approaches have become increasingly important tools for microbiological monitoring. Real-time PCR enables rapid and sensitive detection of microbial DNA and can complement conventional cultivation methods by providing broader insight into microbial community composition [*12*]. Although molecular methods cannot distinguish between viable and non-viable cells [*13*], combining culture-dependent and molecular approaches provides a more comprehensive assessment of contamination events and microbial dynamics than either method alone [*14*].

The integration of environmental and product-level monitoring may improve understanding of contamination routes and support the identification of critical control points (CCPs) within food production systems [*9*]. Environmental monitoring can reveal contamination hotspots within production facilities [*9*], whereas product monitoring enables the evaluation of microbial succession and contamination dynamics throughout individual stages of cheese manufacture and ripening [*15,16*]. Such information is particularly relevant for HACCP-based management systems, where effective risk control depends on accurate identification of contamination sources and transmission pathways [*17*].

Despite increasing knowledge of cheese-associated microbiota, the interactions between environmental reservoirs of contamination and microbial communities developing on smear-ripened cheeses remain insufficiently characterized [*18,19*]. In particular, studies combining environmental surveillance, product-level monitoring, and the evaluation of sanitation interventions under industrial production conditions remain limited. Consequently, evidence supporting the identification of contamination pathways and CCPs in smear-ripened cheese production remains incomplete.

To address these knowledge gaps, the present study integrated environmental and product-level microbiological monitoring within an industrial smear-ripened cheese production facility. Airborne microbiota was evaluated at 24 sampling locations using sedimentation sampling, cultivation, microscopy, and real-time PCR, while microbial succession during cheese manufacture was monitored across ten technological stages using parallel culture-dependent and molecular

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approaches. In addition, the impact of whole-facility ethylene oxide fumigation on environmental contamination and product-associated microbiota was assessed.

The objectives of this study were to: (*i*) characterize airborne microbiota and identify contamination hotspots using culture-dependent and molecular methods; (*ii*) evaluate microbial dynamics throughout cheese production; (*iii*) compare environmental and product-associated microbiota to identify potential contamination pathways; (*iv*) assess the effectiveness of ethylene oxide fumigation in reducing microbial contamination; and (*v*) identify CCPs relevant to HACCP implementation and propose evidence-based measures for improved microbiological control in smear-ripened cheese production.

MATERIALS AND METHODS

Sampling design and process context

Environmental monitoring was conducted in an industrial smear-ripened cheese production facility located in the South Moravian Region, Czech Republic. Airborne and surface contamination were assessed at 24 locations representing major production areas, including curd reception (S1–S2), cold room (S3, S21), curd vat (S4–S5), processing (S6–S10, S13–S16, S18), ripening (S12a–S12b), cooling (S19a–S20b), storage, and packaging (S17). Three independent biological replicates were collected at each location during two sampling periods: April 2025 (before sanitation intervention) and June 2025 (after sanitation intervention). Between sampling periods, the facility underwent whole-facility ethylene oxide fumigation performed by a certified external contractor according to a validated operating protocol.

Airborne microorganisms were monitored using passive sedimentation sampling. Petri dishes containing selective culture media were exposed for 2 h at each sampling location and subsequently processed for cultivation and molecular analyses. Surface samples were collected from equipment, walls, and floors using sterile swabs pre-moistened with sterile physiological saline. Whenever possible, swab samples were collected from comparable surface areas.

Microbial succession during cheese production was evaluated in two industrial batches (B1 and B2). Approximately 60 g of material was aseptically collected at ten technological stages from raw material processing to final packaging. In addition, raw materials from two independent suppliers were analysed. This design resulted in 12 sampling points per batch, with three independent replicate samples collected at each point.

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Culture-dependent analysis

Plate Count Agar (PCA), Nutrient Agar No.2 (NA), Dichloran Medium Base w/Rose Bengal agar (DRBC) (HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India), de Mann, Rogosa, Sharp agar (MRS) (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and Czapek-Dox Modified Agar (CZA) (Condalab, Madrid, Spain) were used for differential cultivation of bacteria, yeasts and molds. Yeasts and molds were enumerated according to ISO 21527-1:2008 [*20*]. Microbial isolates were characterized based on colony morphology, microscopic examination, and Gram staining where appropriate.

Control strains

The following reference strains were used as positive controls: *Bifidobacterium animalis* CCM 4988^T, *Brevibacterium linens* CCDM 200, *Candida vini* CCY 5962, *Geotrichum candidum* CCF 5692, *Kluyveromyces marxianus* var. *lactis* CCY 26-12-6, *Lactobacillus helveticus* CCDM 121, *Lactobacillus plantarum* CCM 7039^T, *Penicillium citrinum* CCF 1812, *Penicillium commune* CCF 1905, *Penicillium chrysogenum* CCF 3209, *Pichia farinosa* CCY 39-4-2, and *Saccharomyces pastorianus* CCY 21-6-3. All strains were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), the Collection of Dairy Microorganisms (CCDM, Tábor, Czech Republic), and the Culture Collection of Fungi (CCF, Prague, Czech Republic).

Isolation of DNA

Genomic DNA was extracted from environmental samples, cheese samples, and reference strains using the NucleoSpin® Microbial DNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Real-time PCR targets

Qualitative real-time PCR was performed using the primer sets listed in Table 1 under the amplification conditions summarized in Table 2. Amplification reactions were carried out using Rotor-Gene (Qiagen, Hilden, Germany) and LightCycler Nano (Roche Diagnostics, Mannheim, Germany) instruments. The PCR reaction mixture contained 12.5 µL SYTO9 qPCR 2× Master Mix (TopBio, Vestec, Czech Republic), 1 µL of each primer (0.2 µM), 1–2 µL of DNA template and nuclease-free water to a final volume of 25 µL. All primer pairs showed PCR efficiencies between 95–105 % and

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R^2>0.98. No-template controls were included in each run. Amplicon specificity was verified by melting-curve analysis and agarose gel electrophoresis.

Environmental samples were evaluated qualitatively and expressed as presence or absence of target microorganisms. Detection frequency was calculated as the proportion of positive detections among all target-location combinations tested. Samples showing characteristic amplification curves and target-specific melting temperatures were considered positive. Quantitative Ct value analysis was performed only for cheese samples and was used for comparative evaluation of relative target abundance between production batches.

Statistical analysis

Microbiological analyses were evaluated in accordance with ISO 21527-1:2008 [*20*]. All analyses were performed in triplicate to verify the reproducibility and reliability of the obtained results. The relative standard deviation was calculated from triplicate measurements using Microsoft Excel for Microsoft 365 (Microsoft Corporation, Redmond, WA, USA) and did not exceed 10 %. Replicate analyses were used to confirm the consistency of detection patterns across sampling locations. The environmental monitoring dataset consisted primarily of qualitative presence/absence observations collected from multiple sampling locations during two monitoring campaigns. Because of the descriptive nature of these data and the limited number of observations at individual sampling locations, inferential statistical analyses were not considered appropriate. The environmental monitoring data were therefore evaluated descriptively to identify microbial distribution patterns, differences between monitoring campaigns, and potential contamination hotspots rather than to test formal statistical hypotheses. For improved visualization and interpretation of microbial distribution within the production facility, the results were presented as heatmaps prepared with the assistance of Claude AI (Anthropic, PBC, San Francisco, CA, USA). The heatmaps were generated from data supplied and verified by the authors. Claude AI was used exclusively to assist with data visualization and was not used to generate experimental data, perform statistical analyses, or independently interpret the results. All numerical values and final visualizations were checked and approved by the authors.

RESULTS AND DISCUSSION

Airborne microbiota and identification of contamination hotspots

Airborne monitoring performed across 24 sampling locations and two monitoring periods revealed pronounced spatial heterogeneity of microbial contamination within the production facility.

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The data were interpreted primarily as qualitative indicators of microbial distribution within the production facility. Accordingly, the results were evaluated descriptively to identify contamination hotspots and areas with increased contamination risk rather than to quantify differences among sampling locations. Culture-dependent analyses identified the highest fungal deposition during form-filling (Step 3), drying and ventilation (Steps 4–5), washing and dripping (Steps 6–7), and packaging operations (Steps 9–10), indicating that these technological stages were associated with elevated airborne fungal contamination.

The lowest fungal deposition was recorded in the curd cold room, suggesting that contamination originated predominantly from the production environment rather than from incoming raw materials. This observation is consistent with reports identifying production environments as important reservoirs of microorganisms associated with cheese manufacture [*9*].

Microscopy and real-time PCR targeting ITS and *BenA* markers confirmed that *Penicillium* spp. were the predominant airborne fungal contaminants, particularly *P. commune*, *P. chrysogenum*, and *P. citrinum*. Species-specific PCR further confirmed the widespread occurrence of *P. commune* throughout the production facility. These findings are consistent with previous studies identifying *Penicillium* spp. among the most common fungal contaminants of cheese production environments [*3-7*]. The repeated detection of these species across multiple production areas and both sampling periods suggests the presence of persistent contamination niches associated with specific technological operations and environmental conditions [*3,6,7*].

Product-associated microbiota dynamics across technological steps and batches

Analysis of cheese samples collected throughout production revealed microbial succession patterns characteristic of smear-ripened cheeses. Yeasts were detected throughout the production process and increased following application of the smearing culture, with the highest abundance observed during the drying stage. Real-time PCR results confirmed progressive colonization of the cheese surface by yeast populations, which facilitate subsequent establishment of smear bacteria through surface deacidification [*2*].

The abundance of *Brevibacterium linens* increased during later technological stages, particularly following drying and washing operations, reflecting the sequential development of smear microbiota commonly observed in surface-ripened cheeses [*19*]. Although bacterial DNA was detected during early production stages, cultivation results indicated that active growth of smear-associated bacteria occurred predominantly during later stages of ripening.

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Overall, the observed succession pattern corresponded to the expected development of smear-ripened cheese microbiota, indicating successful establishment of the ripening community during production.

Comparison of culture-dependent and molecular detection

Differences were observed between culture-dependent and molecular detection of fungal contamination. While fungal growth was rarely observed on culture media beyond the early technological stages, real-time PCR detected fungal DNA in most samples. These observations indicate that fungal DNA remained detectable even when culturable fungal populations were not recovered [*11,13*].

The combined application of cultivation and real-time PCR provided complementary information and improved the assessment of microbial contamination within the production process. Similar observations have been reported in previous studies employing both culture-dependent and molecular techniques for the investigation of food-associated microbial communities [*12,14*].

Comparison of production batches showed lower fungal DNA detection in batch B2, produced after ethylene oxide fumigation, than in batch B1. In contrast, bacterial and yeast populations displayed comparable trends in both batches, suggesting that the sanitation procedure primarily affected fungal contamination while maintaining the development of the desired smear microbiota.

However, it should be noted that several methodological limitations need to be considered when interpreting these findings. Culture-dependent methods may underestimate microbial diversity because only microorganisms capable of growth under the selected cultivation conditions can be detected [*10,11*]. Conversely, real-time PCR provides highly sensitive detection of target DNA but does not distinguish between viable and non-viable microorganisms [*13*]. Consequently, positive qPCR signals should be interpreted as evidence of microbial occurrence rather than direct evidence of active contamination. Passive settle plate sampling reflects microbial deposition onto exposed surfaces and provides a practical overview of airborne contamination; however, it does not permit quantification of absolute airborne microbial concentration. Furthermore, the environmental monitoring strategy was based primarily on qualitative presence/absence data and therefore does not permit statistical inference or reliable quantitative comparison of microbial abundance among sampling locations. Moreover, the results should be interpreted only in the context of the investigated facility, as environmental microbiota and contamination patterns may be highly facility specific. In addition, the monitoring programme covered a defined period and therefore may not fully capture seasonal or long-term temporal variations in microbial contamination patterns. Nevertheless,

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triplicate analyses with a relative standard deviation below 10% confirmed satisfactory reproducibility and reliability of the obtained results. Despite these limitations, the combined application of cultivation and molecular approaches provided a comprehensive assessment of contamination dynamics within the investigated production facility.

Relationship between environmental and product contamination

Integration of environmental and product-level monitoring revealed an association between airborne contamination and fungal detection in cheese samples. Production steps performed in areas with elevated airborne *Penicillium* counts corresponded to stages exhibiting the highest prevalence of fungal DNA, particularly during forming, drying, washing, and packaging.

These observations support airborne transmission as an important contamination pathway during cheese manufacture.

Although smear microbiota limited visible fungal growth, they did not prevent deposition of fungal spores on cheese surfaces. Consequently, fungal DNA remained detectable throughout several technological stages despite the absence of culturable molds. This finding highlights the value of combining environmental and product-level monitoring when evaluating contamination pathways in smear-ripened cheese production.

Effectiveness of ethylene oxide fumigation

Whole-facility ethylene oxide fumigation was associated with reduced fungal contamination in both environmental and product samples during the second monitoring period. Environmental monitoring showed lower microbial detection within enclosed processing areas, while product-level analyses revealed lower fungal DNA levels and reduced recovery of fungal isolates following fumigation.

Nevertheless, environmental recolonization was observed at facility entry points and cold-storage areas. Increased detection of *Penicillium* and *Cladosporium* DNA in these locations suggests continued introduction of microorganisms from external sources, including raw materials and outdoor air. In contrast, contamination levels remained lower within enclosed processing zones.

The reduction observed in cheese samples was more pronounced than the changes detected in environmental qPCR monitoring data. Together, these findings indicate that fumigation reduced product-associated fungal contamination, while recolonization of specific environmental locations remained evident.

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These observations emphasize the importance of continued environmental monitoring, particularly at facility entry points and storage areas identified as potential sources of contamination. As illustrated in Fig. 1 and Fig. 2, environmental qPCR monitoring revealed distinct spatial patterns of microbial detection throughout the production facility before and after ethylene oxide fumigation. The heat-map visualization facilitated identification of contamination hotspots, locations with persistent microbial occurrence, and areas exhibiting reduced or increased detection frequencies following fumigation. Fig. 1 illustrates the distribution and intensity of positive qPCR detections across microorganism categories and sampling locations, whereas Fig. 2 illustrates the direction and magnitude of changes observed between the pre- and post-fumigation monitoring campaigns. As mentioned, the results were evaluated descriptively and interpreted as indicators of microbial occurrence, contamination distribution patterns, and fumigation-associated changes within the production environment.

Critical control points and evidence-based hygiene interventions

Based on the integrated environmental and product-monitoring results, five contamination-prone processing stages relevant to HACCP verification were identified [*17*].

CCP 1 – Forming (Step 3). The first stage at which fresh curd is directly exposed to the production environment.

CCP 2 – Drying and ventilation (Steps 4–5). Areas associated with intensive air circulation and prolonged exposure of cheese surfaces.

CCP 3 – Washing and dripping (Steps 6–7). Humid processing zones where microbial deposition may occur.

CCP 4 – Packaging (Steps 9–10). The final technological stage before product distribution.

CCP 5 – Environmental reservoirs. Brine tanks, shelving systems, and smearing equipment that may contribute to persistence of environmental contamination despite routine sanitation [*17*].

The identified contamination-prone stages corresponded to locations associated with elevated airborne contamination and increased fungal detection in cheese samples, supporting their relevance for HACCP-based contamination control.

Overall, the results indicate that integrated environmental and product-level monitoring can support identification of contamination pathways, assessment of sanitation measures, and implementation of targeted hygiene interventions. The combined use of cultivation and real-time PCR provided complementary information on microbial contamination within the production process [*13,14,17*].

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CONCLUSIONS

Integrated environmental and product-level monitoring identified *Penicillium* spp. as major fungal contaminants associated with smear-ripened cheese production. Airborne contamination was most frequently detected during forming, drying, washing, and packaging operations, indicating that these technological stages may represent important targets for contamination control. Real-time PCR detected fungal DNA more frequently than cultivation, supporting the complementary value of combining molecular and culture-dependent approaches for contamination assessment.

Comparison of monitoring periods indicated reduced fungal contamination following whole-facility ethylene oxide fumigation. Environmental monitoring revealed a heterogeneous spatial response, with lower microbial detection in enclosed processing areas but evidence of recolonization at facility entry points and cold-storage zones. Increased detection of *Penicillium* and *Cladosporium* DNA in these locations suggests continued introduction of environmental contaminants from external sources.

The contamination-prone processing stages identified in this study may provide a practical basis for HACCP verification and targeted hygiene interventions. Although the findings should be interpreted within the methodological limitations of qualitative environmental monitoring, the integrated monitoring approach combining cultivation, real-time PCR, and environmental surveillance proved effective for identifying contamination hotspots, tracking contamination pathways, and supporting microbiological control in smear-ripened cheese production facilities.

Future studies including multiple production facilities, longer monitoring periods, and complementary quantitative approaches would help to further improve understanding of contamination dynamics and strengthen evidence-based contamination control strategies in smear-ripened cheese production.

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ETHICS APPROVAL

No ethical approval was required, as no experiments involving human participants or animals were conducted.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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AUTHORS' CONTRIBUTION

Štěpánka Trachtová conceived and designed the study, performed formal data analysis, supervised the project, validated the results and prepared the original manuscript draft. Eva Vítová conducted the experimental work, provided resources and contributed to methodology development and manuscript revision. Helena Hudečková participated in data curation, investigation, data analysis and manuscript review. All authors read and approved the final version of the manuscript.

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Table 1. Primer sequences and expected amplicon sizes

Target group	Primer	Sequence (5′–3′)	Amplicon size/bp	Reference
Bacteria (domain)	Feub	TCC TAC GGG AGG CAG CAG T	466	[*21*]
	Reub	GGA CTA CCA GGG TAT CTA AT C CTG TT		
Total yeast (a)	Oli_F	CGT CAT AGA GGG TGA GAA T CC	152	[*22*]
	Oli_R	ACT TGT TCG CTA TCG GTC TC		
Total yeast (b)	NL1F	GCA TAT CAA TAA GCG GAG G AA AAG	680	[*23*]
	NL4R	GGT CCG TGC AAG ACG G		
Candida	CandUn	CAT GCC TGT TTG AGC GTC	251	[*24*]
	FungUn	TCC TCC GCT TAT TGA TAT GC T		
Kluyveromyces	18SBDir	GCG ATA ACG AAC GAG ACC TTA A	251	[*25*]
	18Sdinv	TTG AGC CAT AAT TGG TCT		
Brevibacterium linens	68	TGC CTC AGA TTG AAC GCT G GC GGC	1470	[*26*]
	1450	CCT TGT TAC GAC TTA GTC CC A AT		
Geotrichum candidum	GcandiF	AGA TTG TAT CTT GAG AGC GG A TTA	299	[*24*]
	GcandiR	GCC GAA ACA CAG TTG AAC A A		
Penicillium commune	COMF	CCC GTG TTT ATT TTA CCT TG	464	[*27*]
	COMR	CTG GAT AAA ATT TGG GTT GA		
Total moulds (*Fungi*-β-tubulin, *BenA* gene)	BT2a	GGT AAC CAA ATC GGT GCT G CT TTC	475–562	[*28*]
	BT2b	ACC CTC AGT GTA GTG ACC CT T GGC		
ITS region	ITS1	TCC GTA GGT GAA CCT GCG G	630–840	[*29*]
	ITS4	TCC TCC GCT TAT TGA TAT GC		
	ITS5	GGA AGT AAA AGT CGT AAC AAG G	315	
	ITS2	GCT GCG TTC TTC ATC GAC G		

Note: ITS = Internal Transcribed Spacer; bp = base pairs

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Table 2. Real-time PCR cycling conditions used in this study

Primer set	Denaturation		Annealing		Extension		*N*(cycle)
	Temperature/°C	*t*/s	Temperature/°C	*t*/s	Temperature/°C	*t*/s	
Feub/Reub	95	10	55	30	72	30	30
Oli_F/Oli_R	94	60	51	30	72	60	30
NL1F/NL4R	94	20	55	20	72	20	40
CandUn/FungUn	95	30	58	30	72	30	35
18SBDir/18Sdinv	95	45	60	45	72	45	35
68/1450	92	30	57	50	72	60	35
GcandiF/GcandiR	95	30	60	30	72	30	35
COMF/COMR	95	30	55	30	72	30	30
BT2a/BT2b	94	45	60	45	72	60	35
ITS1/ITS4, ITS5/ITS2	95	45	55	45	72	60	35

Note: Initial denaturation at 95 °C for 300 s and final extension at 72 °C for 300 s were identical for all assays, with real-time PCR reactions performed using Rotor-Gene Q (Qiagen, Hilden, Germany) and LightCycler Nano (Roche Diagnostics, Mannheim, Germany)

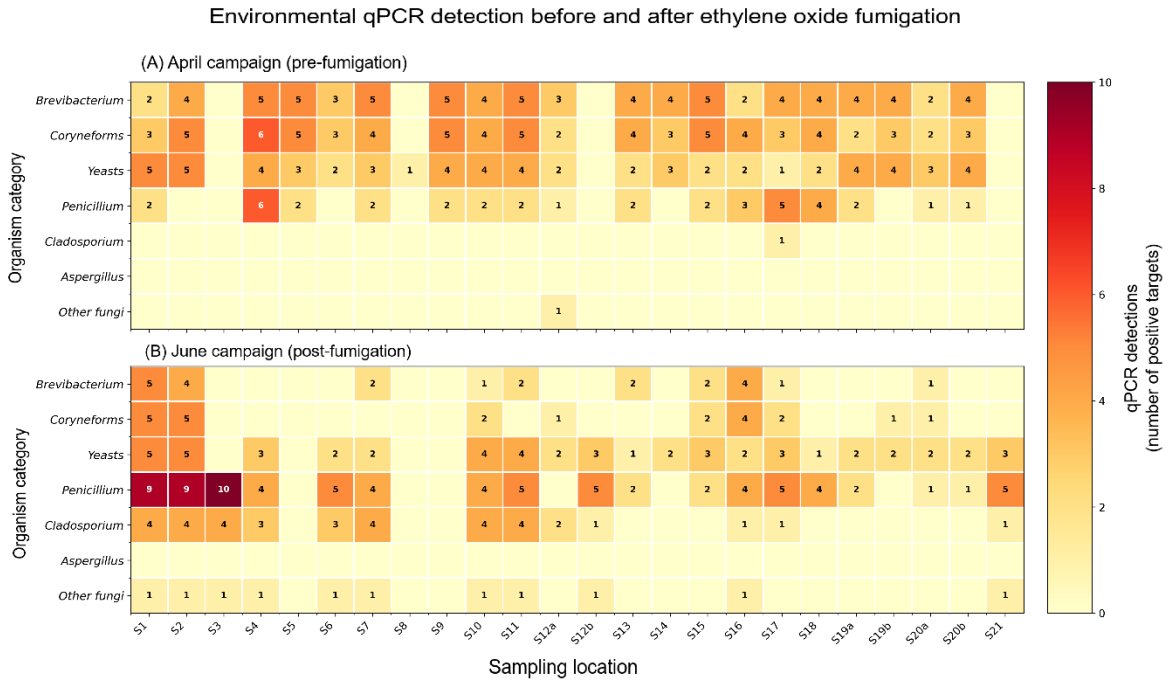


Fig. 1. Environmental qPCR detection before and after ethylene oxide fumigation. The figure presents two heatmaps displaying the results of qualitative real-time PCR (qPCR) environmental monitoring conducted at 24 sampling locations (S1–S21, with S12a/b, S19a/b, and S20a/b as split sites) across an industrial smear-ripened cheese production facility. Panel (A) shows data from the April campaign (pre-fumigation) and panel (B) from the June campaign (post-fumigation). The y-axis categorizes

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detected organisms into seven groups: *Brevibacterium*, coryneforms, yeasts, *Penicillium*, *Cladosporium*, *Aspergillus*, and other fungi. Each cell displays the number of positive qPCR targets detected at that location–organism combination, with colour intensity ranging from pale yellow (low detection, 0–2) through orange to deep red/dark crimson (high detection, up to 10), as indicated by the colour scale on the right.

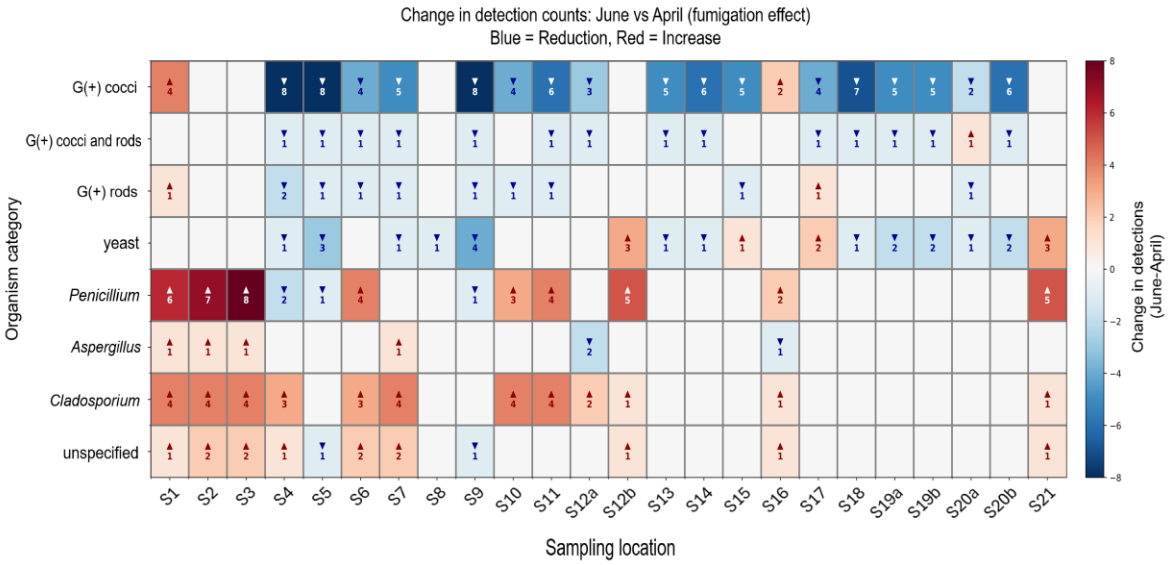


Fig. 2. Environmental DNA distribution by organism category (simplified difference). The heatmap visualizes the net change in qPCR detection counts between the post-fumigation (June) and pre-fumigation (April) campaigns, calculated as June minus April for each organism category at each of the 24 sampling locations (S1–S21, including split sites S12a/b, S19a/b, S20a/b). The colour scale is bipolar: blue cells with downward-pointing triangles (▼) indicate a reduction in detection counts following fumigation, while red cells with upward-pointing triangles (▲) indicate an increase. Colour intensity reflects the magnitude of change, ranging from dark navy blue (reduction of up to −8) through pale tones (minimal change) to dark crimson red (increase of up to +8). Eight organism categories are represented on the y-axis: G(+) cocci, G(+) cocci and rods, G(+) rods, yeast, *Penicillium*, *Aspergillus*, *Cladosporium*, and unspecified