Justus-Liebig-Universität Gießen

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"Hatchery Workers' Exposure to Airborne Bacteria and Development of Immunofluorescence Based Methods for Detection of Antibody Responses"

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Tag der Disputation:	26. April 2017

Content

I Declaration of Originality

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„Ich erkläre: Ich habe die vorgelegte Dissertation **Hatchery Workers' Exposure to Airborne Bacteria and Development of Immunofluorescence Based Methods for Detection of Antibody Responses** selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der ‚Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis' niedergelegt sind, eingehalten."

Berlin, den 28.01.2017

Paul Brauner

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III Summary

Structural changes in livestock farming also led to densification of poultry egg production in commercial hatcheries. In consequence work conditions altered significantly and hatchery workers are frequently exposed to bioaerosols that are insufficiently characterized. Moreover, etiology of occupational bioaerosol-related respiratory disorders is not well understood. In this doctoral thesis quantitative and qualitative culture dependent and independent methods were applied to analyze and to compare in particular airborne bacterial communities in different poultry hatcheries. Furthermore, the source of airborne bacteria in hatcheries was investigated. Considering that bioaerosol-related health effects and disorders often share immunological features this study also aimed at development of adequate serological methods for detection of workers' specific immunoreactions to workplace antigens.

Determination of the microbial exposure in a chicken and a turkey hatchery during different tasks with handling of poults or eggshells revealed high concentrations of airborne microorganisms (up to 5.3 x 10^6 cells m^{-3}). Analysis of bacterial community compositions by construction of clone libraries displayed a low species diversity but an extensive similarity on the genus level in both hatcheries, revealing *Enterococcus*, *Clostridium*, *Staphylococcus* and *Acinetobacter* as predominant genera. Moreover, identification of bacterial and fungal isolates confirmed occurrence of various pathogens which are classified as risk group 2 microorganisms (German technical rule for biological agents (TRBA) 460, 466). Furthermore, the bacterial community present on eggshells samples was analyzed. For the first time it was documented that eggshell fragments facilitate excessive bacterial growth under hatchery incubator conditions and multiplication clearly contributes to potential harmful bioaerosol formation.

Determination of workers antibody titers against specific occupational microbial antigens may lead to identification of potentially harmful species. Since indirect immunofluorescence (IIF) is easy to implement, this technique was used to analyze immunoreactions in human sera. In order to address disadvantageous inter-observer variations as well as the absence of quantifiable fluorescence data in conventional titer determination by eye, a tool for automated image analysis was developed and validated. The *Fluorolyzer* soſtware is able to reliably quantify fluorescence intensities of antibody-bound bacterial cells on digital images. Subsequently, fluorescence values of single cells have been used to calculate non-discrete IgG titers to bacterial isolates from duck hatchery air. Moreover, in addition to established assays with pure bacterial cultures, a new approach utilized complex bioaerosol samples for detection of anti-microbial antibodies in human sera by determination of percentages of

antibody-bound cells in different serum dilutions. Mean titers in sera from hatchery workers and a non-exposed control group did not display significant differences for most tested isolates and application of comprehensive cluster analysis to entire titer data revealed no structure reflecting workers and controls group. Furthermore, determination of immunoreactivity to the complete microbial community in workplace air displayed similar proportions of antibody-bound cells in both groups. Although no general differences in immunoreaction patterns were observed, mean titers to a *Proteus mirabilis* isolate and to 3 of 4 distinct *Acinetobacter baumannii* isolates were higher in the group of hatchery workers than in the reference group indicating a need for further investigations regarding potential involvement in pathogenesis of occupational respiratory disorders.

A novel approach presented here aimed at identification of immunogenic bacteria in complex bioaerosol samples. For this purpose a protocol for separation of bacterial cells from complex bioaerosol samples by Laser Microdissection and Pressure Catapulting (LMPC) was developed. Although technical key issues like an appropriate base material or mode of cell lysis have been established successfully, PCR based amplification of 16S rRNA genes from microdissected cells could not be accomplished until now and remains to be further optimized.

IV List of Scientific Manuscripts

Results presented in this doctoral thesis were published in international scientific journals (II, III, IV) or were documented in hitherto unpublished manuscripts (I, V).

I Occupational Exposure to Airborne Bacteria in Different Poultry Hatcheries

II Paul Brauner, Kerstin Klug and Udo Jäckel. Eggshells as a Source for Occupational Exposure to Airborne Bacteria in Hatcheries. *Journal of Occupational and Environmental Hygiene*, 13.12 (2016): 950-959.

III Paul Brauner and Udo Jäckel. Automated Image Analysis for Determination of Antibody Titers Against Occupational Bacterial Antigens Using Indirect Immunofluorescence. *The Annals of Occupational Hygiene*, 60.5 (2016): 643-648

IV Paul Brauner, Silvana Gromöller, Yvonne Pfeifer, Gottfried Wilharm and Udo Jäckel. Hatchery Workers‘ IgG Antibody Profiles to Airborne Bacteria. *International Journal of Hygiene and Environmental Health*, Article in Press, available online 26 December 2016

V Use of Laser Microdissection for Identification of Immunogenic Bacteria in Bioaerosols

1 REVIEW

Over the last decades German agriculture faced a fundamental structural change, marked by a tremendous increase in production and a development from traditional farming towards factory farming. In the last 15 years German poultry industry displayed a massive growth in meat production. For example, poultry slaughter exhibited a 97% increase from year 2000 until 2015 gaining 1.5 million tons of meat per year (Federal Statistical Office, Germany, 2016). Simultaneously, animal husbandry passed a process of intense densification: the number of animals in a single poultry farm increased to an average of 2.100 (Federal Statistical Office, Germany, 2010) and poultry confinement changed to high stocking densities with up to 26 broilers sharing one square meter (Federal Ministry of Food and Agriculture, Germany, 2014). Densification also affected hatcheries, the starting point of the poultry production chain (Fig. 1). In 2004 103 German hatcheries had a combined capacity for approx. 57 million eggs. Only 10 years later the capacity has increased to 85 million eggs although the number of hatcheries declined to 69 (Federal Statistical Office, Germany, 2015). These extensive changes had a deep impact on work conditions at all stages of poultry production and facilitate occurrence of complex bioaerosols containing viruses, bacteria, endotoxin, fungi, spores (Hagmar et al. 1990; Lacey and Dutkiewicz 1994). In particular high concentrations of airborne bacteria are frequently detected and a cause for concern (Radon et al. 2002; Fallschissel et al. 2010; Lawniczek-Walczyk et al. 2013). However, associations of occupational exposure to massive microbial air contamination and potential health risks are rarely characterized.

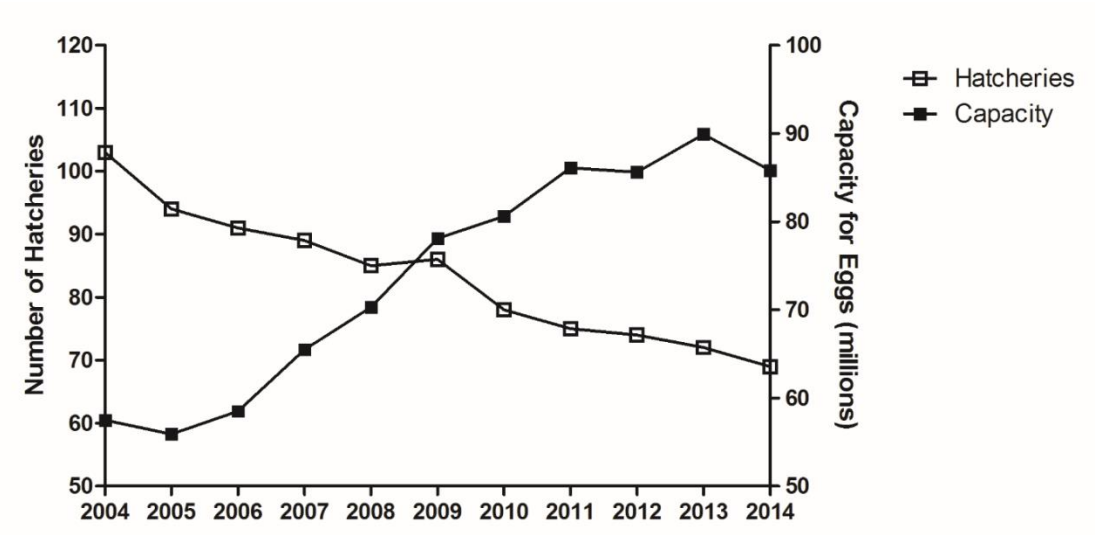

Figure 1 Number of German hatcheries and the corresponding capacity for hatching eggs from 2004 to 2014 (Federal Statistical Office, Germany, 2015).

1.1 Airborne Bacteria in Poultry Production

In comparison to other livestock facilities, higher levels of microbial air contamination were detected in poultry husbandry (Seedorf et al. 1998; Radon et al. 2002; Bakutis, Monstviliene and Januskeviciene 2004) where several potential sources for airborne bacteria are present. Next to feathers that are covered with up to 7 x 10^5 cfu cm^{-2} (Morar et al. 2008), feces contains large quantities of microorganisms (up to 10^{11} cells g^{-1}) (Gong et al. 2002) and especially poultry wing beating facilitates the release into workplace air (Saleh, Seedorf and Hartung 2003). Bacteria in poultry bioaerosols are attached to dust particles or suspended freely in the air (Whyte 1993) and concentrations up to 10^8 cells m^{-3} were detected during chicken catching (Nielsen and Breum 1995) or up to 10^9 cells m^{-3} in poultry confinement houses (Radon et al. 2002). Those notably high numbers exceed cell counts in normal outside air (approx. 10^4 cells m^{-3}) by several orders of magnitude (Bovallius et al. 1978; Bauer et al. 2002; Maron et al. 2005). Analyses of bacterial communities in poultry houses displayed a large taxonomic diversity and gram positive genera as *Staphylococcus*, *Micrococcus*, *Enterococcus*, *Streptococcus*, *Aerococcus*, *Corynebacterium*, *Lactobacillus* as well as *Bacillus* were found being predominant (Sauter et al. 1981; Radon et al. 2002; Vučemilo et al. 2007; Fallschissel et al. 2010; Just et al. 2011; Lawniczek-Walczyk et al. 2013). Furthermore, genera comprising important gram negative foodborne pathogens as *Campylobacter*, *Salmonella* or *Escherichia* are frequently identified in poultry houses (Whyte et al. 2001; Chinivasagam et al. 2009; Fallschissel, Kämpfer and Jäckel 2009; Ellis-Iversen et al. 2012).

However, next to workers in poultry houses also employees in corresponding slaughtering plants are exposed to highly concentrated poultry derived bioaerosols. Moreover, concentrations of airborne bacteria are significantly higher at final stages of fattening and adult animals are supposed to be massively contaminated (Vučemilo et al. 2007; Oppliger et al. 2008). Levels of airborne bacteria detected in such facilities are similar to those in poultry houses as Ellerbroek et al. (1997) detected up to 10^8 cfu m^{-3} total bacteria. Furthermore, bacterial genera identified in different slaughtering plants comprise a similar spectrum as in poultry houses. Next to predominant *Staphylococcus*, *Micrococcus*, *Bacillus* and *Corynebacterium* also various gram negative genera as *Moraxella*, *Acinetobacter*, *Klebsiella* and *Pseudomonas* were found (Ellerbroek 1997; Lues et al. 2007; Liang et al. 2013).

In contrast to poultry houses and slaughter houses, hatcheries appear “cleaner”, most likely due to the absence of adult animals, feed, litter and feces. Thus, issues of microbial contamination in hatcheries rather concerned prevention of hatching egg infection by *Salmonella* spp. (Bailey et al. 1996; Berrang et al. 1999) or other gram negative bacteria

(Gordon and Ahmad 1993) than occupational exposure. According to that only few studies reported on workplace bioaerosols in hatcheries and potential harmful effects of the specific microflora are a largely unresearched field. Nevertheless, pioneer investigations on air contamination in polish chicken hatcheries by Dutkiewicz (1980) and further research by Chmielowiec-Korzeniowska et al. (2007) as well as Tymczyna et al. (2007) revealed elevated loads of bacteria exceeding 10^5 cfu m^{-3} and occurrence of well-known clinical pathogens including *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Staphylococcus aureus* (Tab. 1). Analysis of work place bioaerosols in a duck hatchery by Martin et al. (2011) using molecular methods confirmed these results to a large extent and displayed that major fractions of airborne bacteria could be assigned to only three genera: *Staphylococcus*, *Enterococcus* and *Acinetobacter*. Moreover, in a following duck hatchery study (Martin et al. 2013) workers' personal microbial exposure at days with and without duckling processing was monitored and an approx. 1000-fold higher microbial cell concentration in workplace air was determined when workers handled freshly hatched ducklings. However, the few available studies aiming at characterization of entire bacterial communities in hatchery air focused on facilities that processed chicken or duck hatching eggs only. Until now, the influence of different poultry species on bioaerosol exposure in hatcheries has not been analyzed with an identical technical approach using cultivation dependent and independent methods. Furthermore, the specific exposure in turkey hatcheries has not been characterized yet. This lack of comparative data led to investigations in a chicken and a turkey hatchery (Manuscript I). Accounting for 90.8% (chicken) and 6.4% (turkey) of total hatching eggs these two poultry species represented the vast majority of German poultry production in 2014 (Federal Statistical Office, Germany, 2015). As reported in the aforementioned Polish chicken and German duck hatchery studies also in the recently investigated chicken and turkey hatcheries employees are exposed to high concentrations of airborne bacteria. In a novel approach using task based sampling, individual exposure during different work activities with and without poult handling has been compared. In both investigated hatcheries levels of mesophilic airborne bacteria were clearly higher during poult processing at days with regular removal from hatcher incubators than at days without poult removal and processing or in outside air. However, comparison of levels of airborne microorganisms detected during different tasks including initial sorting (Figure 2), debeaking or sexing of poults revealed no substantial differences.

Figure 2 Hatchery workers sorting turkey hatchlings immediately after removal from incubators.

Results from cultivation and total cell count displayed higher bacterial air contamination in the chicken hatchery gaining 5.4 x 10^5 cfu m^{-3} and 5.3 x 10^6 cells m^{-3}, respectively. These numbers are comparable to those reported from chicken hatcheries (Chmielowiec-Korzeniowska et al. 2007; Bródka et al. 2012) but lower than levels determined in a duck hatchery by Martin et al. (2011) as they detected 7 x 10^6 cfu m^{-3} and 2 x 10^7 cells m^{-3}. Hence, next to poultry specific differences, other factors, including type of ventilation as well as temperature and air humidity might also significantly influence microbial concentrations. Analysis of the composition of bacterial communities in chicken and turkey hatchery air by generation of 16S rRNA gene clone libraries and identification of isolates revealed an extensive similarity on the genus level and in species richness. However, compared to the greater bacterial diversity determined in turkey (28 genera) (Fallschissel et al. 2010) and duck houses (29 genera) (Martin, Kämpfer, and Jäckel 2010) by the same methodological approach, in the here investigated chicken and turkey hatcheries only 18 and 20 genera, respectively, were counted. Likewise, Martin et al. (2011) detected 13 genera in duck hatchery air. In conclusion, hatcheries display a lower diversity than poultry houses and these findings might indicate less sources for bacterial contamination and more homogenous habitats. In both hatcheries *Enterococcus*, *Clostridium*, *Staphylococcus* and *Acinetobacter* were identified as predominant genera. While strictly anaerobic *Clostridium* species were not detected by solely aerobic cultivation by Chmielowiec-Korzeniowska et al. (2007) occurrence of identical genera in duck, chicken and turkey hatchery air indicates the presence of similar

ecological conditions independently from the poultry species. Furthermore, in contrast to other hatchery studies, this report also aimed at quantification and identification of mould isolates recovered from hatchery air. However, concentrations in hatchery air did not differ substantially from those in outside air and abundant isolates in both hatcheries were identified as species of the genera *Aspergillus* and *Penicillium* which were also identified as predominant fungi in broiler houses (Vučemilo et al. 2007).

Although microbial air contamination in hatcheries is apparently associated to processing of hatchlings, original sources and potential formation mechanisms remained unclear. In particular, since hatcheries are subject of intensive cleaning and disinfection after processing of each batch of hatching eggs as well as bactericidal treatment of hatching eggs before entry into the hatchery (Berrang et al. 2000). A first effort to identify major bacterial sources was made by Martin et al. (2013). They reported identical restriction fragment length polymorphism (RFLP) patterns of restricted 16S rRNA gene amplicons generated from bacterial communities on ducklings fluff and in workplace air. However, since hatchlings are supposed to be uncontaminated as long as they did not emerge from eggs, these findings most likely are a consequence of contamination by a primary source. A new aspect was added to this issue when bacterial multiplication on shell fragments of fresh hatching eggs was identified as a major source for bioaerosol formation in a turkey hatchery (Manuscript II). Although the bacterial colonization and infection of avian eggs was already investigated to a large extent (Board and Tranter 1995), the authors documented for the first time that interiors of cracked eggshells and high temperatures as well as high relative humidity present in incubators during thirty hours of hatching provide appropriate conditions for excessive growth of certain species that has been entrapped in porous shell structures and escaped from hatching egg sanitation. Airflows inside incubators, poult activity as well as transport and handling of contaminated equipment and waste are supposed to cause a release of bacteria into hatchery atmosphere (Fig. 3).

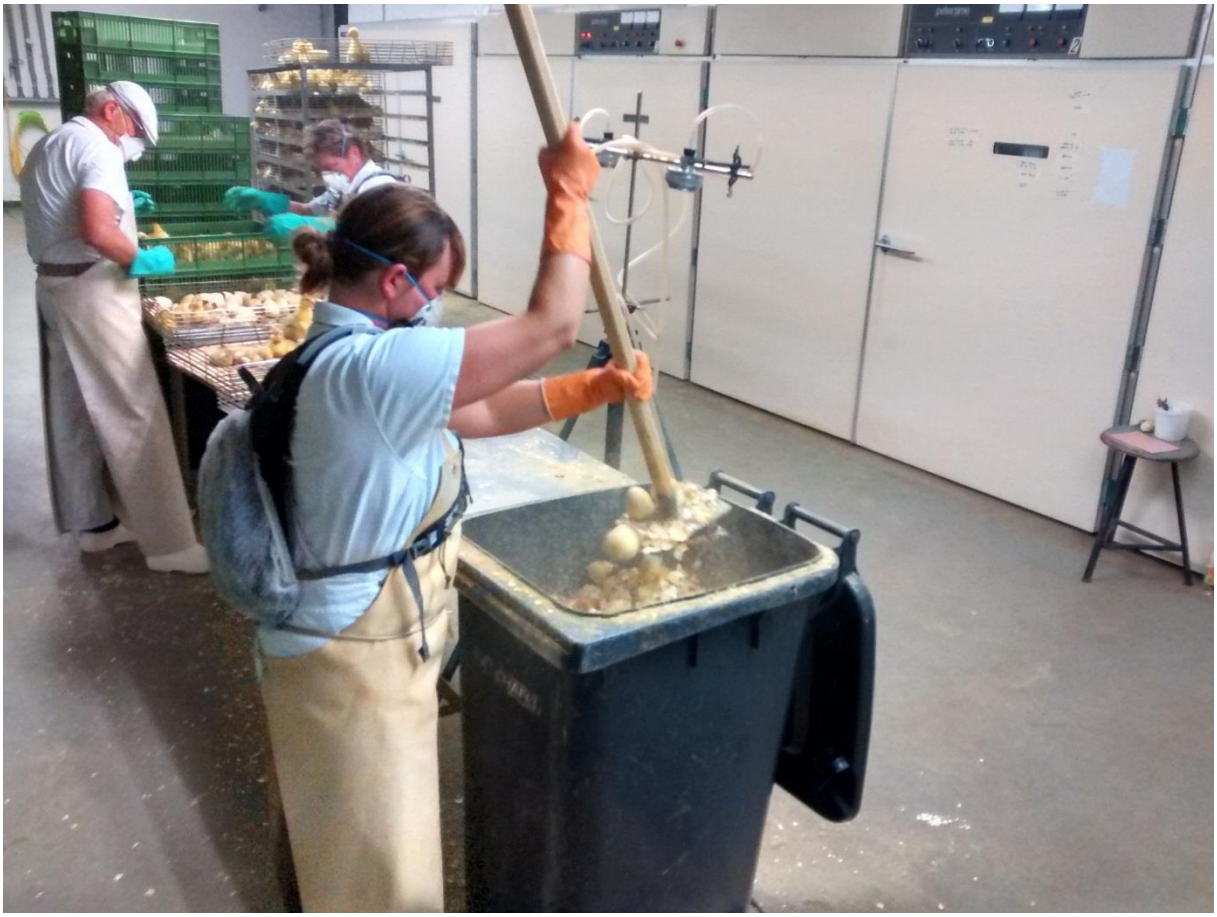

Figure 3 Bioaerosol formation during waste processing.

Moreover, in this study *Enterococcus* was identified as predominant genus on turkey eggshells and in workplace air during poult processing, demonstrating turkey hatching eggs as vehicle for a specific microflora that is transferred from breeder flocks to hatcheries and that contributes to bioaerosol formation. In consideration of these results an intensive hatching egg sanitization apparently does not prevent occurrence and multiplication of pathogens and so does not crucially contribute to a higher occupational safety level. On the contrary a reduced application of disinfectants might alleviate health risks emerging from frequent chemical exposure.

Next to issues of occupational exposure in livestock farming also issues of public health have to be considered since such facilities emit bioaerosols in surrounding environments. Investigations by Baykov and Stoyanov (1999) indicated a dispersal in a distance of up to 3000 m from emitting buildings. Thus, an exposure of the population living in close distance to production plants is possible. In particular, poultry production seems to be more relevant than others as Seedorf et al. (1998) compared emissions of viable bacteria from different livestock buildings and detected highest rates for broilers farms. These results were supported by Gärtner et al. (2014) when they determined emission rates up to 7×10^9 cells per second in

eight different broiler houses. Major bacterial emission fractions consist of members of the genus *Staphylococcus* (Chinivasagam et al. 2010; Gärtner et al. 2011; Martin et al. 2012) and their applicability as marker organisms for emission monitoring was proposed (Schulz et al. 2004; Chinivasagam et al. 2010). Moreover, investigations on emissions of livestock-associated methicillin-resistant *S. aureus* (MRSA) from poultry barns revealed a spread in soils and ambient air (Friese et al. 2013 (a); Friese et al. 2013 (b)) indicating potential risks for colonization of local residents and personnel (Bisdorff et al. 2012; Geenen et al. 2013).

Table 1 Bacterial genera identified in hatchery air.

Genus	Poultry species	References
Acinetobacter	chicken, duck	Dutkiewicz 1980; Chmielowiec-Korzeniowska et al. 2007; Martin & Jäckel 2011; Martin et al. 2013
Achromobacter	duck	Martin et al. 2013
Agrobacterium	chicken	Chmielowiec-Korzeniowska et al. 2007
Alcaligenes	chicken	Chmielowiec-Korzeniowska et al. 2007
Aureobacterium	chicken	Chmielowiec-Korzeniowska et al. 2007
Bacillus	chicken, duck	Chmielowiec-Korzeniowska et al. 2007; Martin & Jäckel 2011; Martin et al. 2013
Brevibacterium	chicken	Chmielowiec-Korzeniowska et al. 2007
Brevundimonas	chicken	Chmielowiec-Korzeniowska et al. 2007
Chlamydophila	guinea fowl, chicken, turkey	Dickx & Vanrompay 2011
Chryseobacterium	chicken, duck	Chmielowiec-Korzeniowska et al. 2007; Martin & Jäckel 2011
Citrobacter	chicken	Chmielowiec-Korzeniowska et al. 2007
Clostridium	duck	Martin & Jäckel 2011; Martin et al. 2013
Corynebacterium	chicken	Chmielowiec-Korzeniowska et al. 2007
Dermacoccus	chicken	Chmielowiec-Korzeniowska et al. 2007
Enterobacter	chicken, duck	Chmielowiec-Korzeniowska et al. 2007; Gehan 2009; Martin et al. 2013
Enterococcus	chicken, duck, turkey	Dutkiewicz 1980; Chmielowiec-Korzeniowska et al. 2007; Martin & Jäckel 2011; Martin et al. 2013; Brauner et al. 2016
Empedobacter	chicken	Chmielowiec-Korzeniowska et al. 2007
Escherichia	chicken, duck	Chmielowiec-Korzeniowska et al. 2007; Gehan 2009; Martin & Jäckel 2011
Gordonia	chicken	Chmielowiec-Korzeniowska et al. 2007
Janthinobacterium	duck	Martin et al. 2013
Jonesia	chicken	Chmielowiec-Korzeniowska et al. 2007
Klebsiella	chicken, duck	Chmielowiec-Korzeniowska et al. 2007; Gehan 2009; Martin et al. 2013
Leclercia	chicken	Chmielowiec-Korzeniowska et al. 2007
Macrococcus	duck	Martin et al. 2013
Microbacterium	chicken	Chmielowiec-Korzeniowska et al. 2007
Micrococcus	chicken	Chmielowiec-Korzeniowska et al. 2007
Mycobacterium	duck	Martin & Jäckel 2011
Pantoea	chicken	Chmielowiec-Korzeniowska et al. 2007
Pediococcus	chicken	Chmielowiec-Korzeniowska et al. 2007
Proteus	chicken, duck	Gehan 2009; Martin & Jäckel 2011
Pseudomonas	chicken, duck	Chmielowiec-Korzeniowska et al. 2007; Gehan 2009; Martin & Jäckel 2011; Martin et al. 2013
Pusillimonas	duck	Martin & Jäckel 2011
Rhodococcus	chicken	Chmielowiec-Korzeniowska et al. 2007
Saccharopolyspora	chicken	Chmielowiec-Korzeniowska et al. 2007
Salmonella	chicken	Cason et al. 1994; Chmielowiec-Korzeniowska et al. 2007; Gehan 2009
Sphingobacterium	chicken	Chmielowiec-Korzeniowska et al. 2007
Sphingomonas	chicken	Chmielowiec-Korzeniowska et al. 2007
Staphylococcus	chicken, duck	Dutkiewicz 1980; Chmielowiec-Korzeniowska et al. 2007; Martin & Jäckel 2011; Martin et al. 2013
Stenotrophomonas	duck	Chmielowiec-Korzeniowska et al. 2007; Martin & Jäckel 2011
Streptococcus	chicken	Chmielowiec-Korzeniowska et al. 2007
Streptomyces	chicken, duck	Chmielowiec-Korzeniowska et al. 2007; Martin & Jäckel 2011
Thermoactinomyces	chicken	Chmielowiec-Korzeniowska et al. 2007
Thermomonospora	chicken	Chmielowiec-Korzeniowska et al. 2007

1.2 Quantification and Identification of Airborne Bacteria

According to German legislation (Act on the Implementation of Measures of Occupational Safety and Health to Encourage Improvements in the Safety and Health Protection of Workers at Work) employers are obliged to establish a specific risk assessment considering any kind of occupational hazards including biohazards. For work activities with biological agents requirements for an adequate risk assessment are stated in the "Biological Agents Ordinance". It demands procurement of detailed information concerning microorganisms' identity and potential infectiousness, transmission paths as well as quality and duration of employees' exposure. Although establishment of a specific risk assessment does not include a mandatory quantitative or qualitative determination of the actual airborne microflora, it is useful to conduct such measurements in order to compare different exposure situations, to detect effects of protective measures or to investigate workplaces in cases of occupational disease (Albrecht, Kiel and Kolk 2007). Albeit first efforts towards standardization were made with the establishment of recommendations for quantification of airborne bacteria at workplaces (IFA Folder 9430) by the German Institute for Occupational Safety and Health (IFA) of the German Social Accident Insurance (DGUV), until now there are no standards comparable to those already existing for environmental measurements in German VDI (Association of German Engineers) guidelines. In fact, several different technical procedures for sampling (e.g. impaction, impingement, filtration) and sample processing are currently applied (Albrecht, Kiel and Kolk 2007). Moreover, existing data on concentrations and identity of airborne bacteria in poultry production often originate from different sampling strategies and technical approaches, thereby impairing the comparability of results.

Conventional quantification of airborne bacteria is based on cultivation of sampled microorganisms. For quantification of total bacteria unselective culture media are used and especially casein-soy-peptone agar is recommended in VDI guideline 4253 part 3. Next to unselective media also selective culture media are used for cultivation and quantification of single species or species groups (Zucker, Trojan and Müller 2000; Venter, Lues and Theron 2004). This type of media also includes media containing chromogenic substrates that target bacterial enzymes for differentiation of pathogens in polymicrobial cultures (Perry and Freydiere 2007). However, commercially available selective media often have been developed for cultivation of a relatively small spectrum of bacteria that are relevant in clinical diagnostics or in food analysis. Their applicability for quantitative analysis of specific bacteria

in highly diverse bacterial communities occurring in animal husbandry is limited due to co-cultivation of non-target species (Gärtner et al. 2014).

Generally, cultivation based techniques suffer from major drawbacks when aiming at quantification of microbial air contamination. Unknown demands for cultivation conditions of many species (Amann, Ludwig and Schleifer 1995) as well as desiccation stress during sampling, transport or storage may lead to death or "viable-but-not-culturable" (VBNC) state (Heidelberg et al. 1997; Mohr et al. 2016) and causes significant underestimation of actual levels of airborne bacteria (Chi and Li 2006; Martin and Jäckel 2011). For these reasons, approaches like cultivation independent 4',6-diamidino-2-phenylindole (DAPI) staining of bioaerosol samples provide more reliable quantification (Albrecht et al. 2007). However, only few studies report total cell counts from work places in poultry production, although results from colony count and total cell count can differ by several orders of magnitude (Martin, Kämpfer and Jäckel 2010; Martin and Jäckel 2011). As further cultivation independent techniques quantitative Real-Time PCR (qRT PCR) has been used for quantification of single genera like *Salmonella* (Fallschissel, Kämpfer and Jäckel 2009) and *Staphylococcus* (Oppliger et al. 2008) or flow cytometry and fluorescent *in situ* hybridization (FISH) were applied for quantification of total bacteria (Chi and Li 2006). Also for identification of bacteria cultivation dependent and independent approaches are available. Conventional identification is based on isolation of pure cultures and subsequent analysis of biochemical characteristics, for example by using the Analytic Profile Index (API) systems (Zucker, Trojan and Müller 2000; Chinivasagam et al. 2010; Bródka et al. 2012; Lawniczek-Walczyk et al. 2013). Such systems often have been established for identification of common clinical pathogens. Thus, their reliability for analysis of diverse bacterial communities in environmental habitats can be limited by variability of expression characteristics (Drancourt et al. 2000). Nonetheless, phenotyping is a useful addition if 16S rRNA sequences for two or more species are very similar and sequence analysis fails in differentiating (Moore et al. 2006). Currently, analysis of variable regions in highly conserved 16S rRNA genes is widely used and allows taxonomic classification of prokaryotes (Ludwig and Klenk 2001; Tindall et al. 2010). In addition, results from 16S rRNA sequence analysis provide a higher degree of accuracy for identification of isolates than phenotypic profiling, albeit sample processing is more laborious (Bosshard et al. 2004; Bosshard et al. 2006). Advantageously, 16S rRNA sequence analysis also enables identification of bacteria in environmental samples without cultivation (Amann, Ludwig and Schleifer 1995). In combination with molecular cloning this technique has been successfully applied in bacterial ecology for screening and quantification of 16S rRNA gene

sequences in very different habitats like soil (Dunbar et al. 1999; He, Xu and Hughes 2006), marine environments (Stevens and Ulloa 2008; Zhang, Ki and Qian 2008), the human intestinal tract (Hayashi et al. 2005; Wang et al. 2005) and environmental bioaerosols (Brodie et al. 2007; Urbano et al. 2011). However, despite having enabled new valuable insights into bacterial community composition (Nehmé et al. 2009; Fallschissel et al. 2010; Martin and Jäckel 2011), this promising technique is not yet widely accepted for characterization of workplace bioaerosols in animal husbandry.

1.3 Significance of Bioaerosols for Occupational Health

The internal surface area of the adult human lung exceeds 100 m^2 (Gehr, Bachofen and Weibel 1978), thereby representing a major entrance for biological agents. Moreover, inhalation is the most important way of uptake at many different workplaces (Albrecht, Kiel and Kolk 2007). In contrast to other occupational hazards like chemical substances, biological agents as bacteria and fungi are able to multiply at agricultural workplaces and inside the human organism, making a risk assessment difficult. The situation is even more complicated when employees are exposed to mixtures of biological agents that can change rapidly in concentration and composition. Regarding the host, susceptibility varies to a great extent among individuals. For these reasons the establishment of reliable dose-effect relationships in occupational medicine is problematic and in consequence no limits for biological agents at workplaces were yet defined in Germany (Albrecht, Kiel and Kolk 2007).

Nevertheless, the inhalation of agricultural bioaerosols and in particular those from poultry processing were early found being harmful to workers' health (Emanuel et al. 1964; Boyer et al. 1974; Lenhart and Olenchock 1984). Potential adverse health effects caused by airborne microorganisms are infections, intoxications as well as sensitizations and irritations (Cox and Wathes 1995; Fung and Hughson 2003). Poultry workers frequently reported respiratory symptoms like cough, wheezing and flu-like illness (Radon et al. 2001). Moreover, they exhibit a cross-shift decline of lung function (Rylander and Carvalheiro 2006) and significant dose-response relationships were observed between exposures parameters and pulmonary function decrements or respiratory symptoms (Donham et al. 2000; Viegas et al. 2013; Guillam et al. 2013). In contrast, for hatcheries only little data is available, Martin et al. (2013) reported a clear cross-shift decline in lung function among duck hatchery workers whereas Skórska et al. (2007) detected no decline and only a moderate frequency of work-related symptoms among chicken hatchery workers. Next to other bioaerosol constituents especially cell wall components of gram negative and gram positive bacteria as well as fungi

are supposed to be potent triggers of this complex of symptoms that can range from mild conditions to severe chronic disorders (Douwes et al. 2003; Fung and Hughson 2003; Schneider 2005). Among cell wall polymers, endotoxins of gram negative bacteria, peptidoglycans of gram positive bacteria and fungal β(1,3)-glucans are most prominent (Schütt and Bröker 2011). When binding to pattern recognition receptors like CD14, Toll-like-receptors and β-glucan receptors on cells of the innate immune system such as macrophages and dendritic cells these molecules activate effector mechanisms leading to inflammation (Brown and Gordon 2003; Myhre et al. 2006; Gioannini and Weiss 2007). Based on the underlying inflammatory mechanisms and symptoms for non-infectious diseases, a distinction between allergic and non-allergic respiratory diseases can be made. Non-allergic respiratory disorders reflect types of non-immune-specific acute airway inflammation such as non-allergic asthma, non-allergic rhinitis/mucous membrane irritation syndrome (MMIS) and toxic pneumonitis/organic dust toxic syndrome (ODTS). However, a frequent exposure to bioaerosols facilitates a transition to chronic disorders as chronic bronchitis or chronic obstructive pulmonary disease (COPD) (Douwes et al. 2003). Furthermore, pre-existing conditions or individual host factors (e.g. atopy, smoking) may modify the risk of developing work-related respiratory symptoms (Cullinan et al. 1999; Siracusa et al. 2006). In contrast to non-allergic respiratory symptoms, allergic disorders are pathophysiologic immunoreactions marked by an immune-specific inflammation including a major role of different antibody classes. While allergic rhinitis and asthma are associated with immunoglobulin E (IgE) mediated sensitization (type I allergy), hypersensitivity pneumonitis (extrinsic allergic alveolitis/farmers' lung) is associated to IgG (type III allergy) (Douwes et al. 2003; Fung and Hughson 2003). Hypersensitivity pneumonitis describes a serious pulmonary condition with delayed fever, an influx of inflammatory cells to the lung parenchyma and symptoms resembling those of acute ODTS (Bourke et al. 2001). In agricultural environments fungal antigens of *Aspergillus* and *Penicillium* and bacterial antigens of the thermophilic bacteria *Saccharopolyspora rectivirgula* and *Thermoactinomyces vulgaris* are the most important triggers, although recent studies indicate that antigens of further bacterial species have to be taken into consideration as well (Sennekamp 2013).

Regarding bacterial infections for workers in the poultry supply chain, zoonotic transmission of *Chlamydophila psittaci* has been documented (Gaede et al. 2008; Dickx et al. 2010; Dickx and Vanrompay 2011). Moreover, workers have an elevated risk for *Campylobacter* infections (enteritis) and neurologic manifestations (Guillain-Barré syndrome) might be possible (Wilson 2004; Price et al. 2007 (a); Baker et al. 2012). Infection risks also arise from

temporary or persistent bacterial colonization (Kluytmans, Van Belkum and Verbrugh 1997) and next to pathogenicity also potential antibiotic resistance has to be considered since therapy options might be reduced in cases of occupational infection. In this context occurrence of multi-drug resistant strains of *E. faecalis* (Lemcke and Bülte 2000; Khan et al. 2005), *S. aureus* (Feßler et al. 2011; Friese et al. 2013 (a, b)) and *A. baumannii* (Lupo et al. 2014; Liu et al. 2016) in poultry production adds to this solicitude. For employees in livestock industry nasal carriage is frequently associated to occupational exposure to MRSA (Moodley et al. 2008; Van Cleef et al. 2011) and in particular for poultry workers, farmers and slaughterers carriage of antibiotic resistant strains of *E. coli* or *E. faecalis* has been documented (Van den Bogaard, Jensen and Stobberingh 1997; Van den Bogaard et al. 2001; Price et al. 2007 (b)). Additional risks for occupational infectious and non-infectious respiratory diseases arise from occupational exposure to moulds. Especially for species of the genus *Aspergillus* worker colonization and sensitization to antigens has been reported (Khosravi et al. 2009; Sabino et al. 2012; Cafarchia et al. 2014). Moreover, mycotoxins (e.g. aflatoxin from *Aspergillus*) have been detected in poultry house air and in serum from workers demonstrating occupational exposure (Wang et al. 2008; Viegas et al. 2012). Mycotoxin uptake primarily happens by ingestion via the food chain and by this route an association to liver-cell cancer was confirmed (IARC 2002). Inhalation of contaminated aerosols at agricultural workplaces represents an additional route (Ghosh et al. 1997; Brera et al. 2002), although little is known about potential respiratory health effects caused by fungal toxins.

1.4 Serological Detection of Health Effects

Long term inhalation of agricultural bioaerosols causes a wide range of adverse health effects. However, associations between occupational respiratory disorders and inhalation of complex bioaerosols are not well understood. In particular interactions between a great number of different biological agents and lung tissue, synergistic effects and the precise identity of trigger agents remain to be investigated. Furthermore, unspecific symptoms of respiratory disorders and various modes of origin impair an accurate diagnosis and association to individual occupations. Nevertheless, proven causality of respiratory disorders is a fundamental prerequisite for an approval as occupational disease by the German Social Accident Insurance institutions. In Germany complexes of officially approved occupational diseases (listed in the Occupational Diseases Ordinance) which can be caused by bioaerosols are infectious diseases (Nos. 3101, 3102), diseases caused by organic dust (No. 4201,

hypersensitivity pneumonitis) and obstructive diseases of the respiratory tract (No. 4301, e.g. asthma). However, diagnostic procedures like spirometry do not provide a direct link to single bioaerosol components. Since occupational respiratory diseases often share immunological features, serological approaches utilizing specific antigen-antibody reactions enable detection of immunoreactions against work-related microbial antigens and may help to comprehend etiology. For example, humoral responses to infections typically arise within 10 to 14 days of initial exposure and can persist over years or decades, thus reflecting an individual history of pathogen encounters. In occupational medicine immunological approaches have been early applied to address effects of bioaerosol exposure. Classic examples are immunodiffusion techniques (Ouchterlony and Nilsson 1978). These simple methods provide information about specificity of antigen-antibody binding and allow rough quantification of either antigen or antibody concentration by visualization of immunoprecipitates. By detection of specific antibodies this procedure contributed to identification of antigens involved in Farmers' lung disease (Kobayashi et al. 1963; Pepys et al. 1963). Further examples are skin prick or skin patch tests as an essential procedure to diagnose sensitization to inhalant allergens in IgE-mediated allergic diseases (Zuskin et al. 1994; Rees et al. 1998; Rimac et al. 2010). Currently, sensitive detection and precise quantification of immunoglobulins is performed by using enzyme-linked immunosorbent assays (ELISA) (Luttmann et al. 2014). This approach enables analysis of seroprevalences of different antibody classes either involved in allergic diseases or infections, for example determination of specific anti-bacterial or anti-fungal IgG (Laitinen et al. 1999; Bünger et al. 2000; Tillie-Leblond et al. 2011), specific anti-bacterial or anti-fungal IgE (Scalabrin et al. 1999; Doekes et al. 2004) or total IgE (Skorska et al. 2007; Rimac et al. 2010). However, establishment of an individual ELISA protocol for a single test antigen is time-consuming and it is even more when antibody titers to numerous isolates are intended to be determined. Compared to other test principles, implementation of indirect immunofluorescence (IIF) testing is convenient when using whole-cell antigens (Porsch-Özcürümez et al. 2004), so this technique allows simple and rapid analysis of specific antibody seroprevalences. In clinical routine serology IIF is used for serodiagnosis of infections from *Bartonella henselae* (Amerein et al. 1996), *Coxiella* spp. (Field et al. 2000), *Rickettsia* spp. (Jensenius et al. 2004), *Legionella pneumophila* (Rudbeck, Molbak and Uldum 2008), *Francisella tularensis* (Jenzora et al. 2008), *Chlamydia* spp. (Baud, Regan and Greub 2010), *Leishmania* spp. (Moreno et al. 2014). Commercial suppliers often restrict the range of standardized test antigens to the aforementioned species and antigens from workplace species are in most cases not available (Bünger et al. 2000). While commercial test antigens reflect

species and strains of clinical relevance, their applicability for field studies is limited due to the diversity of antigenic profiles observed in environmental bacterial species and strains which may lead to unreliable and imprecise detection. Although antigen preparation and assay procedure is simple, conventional IIF titer determination by eye relies on the experience of the operator. Moreover, the manual readout suffers from major drawbacks like significant inter-observer variations, difficult interpretation of borderline samples and absence of quantitative fluorescence data (Luger and Krauss 1990; Bakken et al. 1992; Peeling et al. 2000; Wilkinson, Jansen and Van der Waaij 2003; Chiaro et al. 2011). Therefore, approaches for an instrumental quantification have been developed early. Taylor et al. (1974) used a photomultiplier tube for objective fluorescence measurement of emitted light from indirect immunofluorescence. Likewise dissatisfied with conventional IIF evaluation Apperloo-Renkema et al. (1991) demonstrated first that serum antibody titers against *Enterobacteriaceae* were determined more accurately via software-based image analysis rather than by a human investigator. Both issues, a lack of commercially available test systems and disadvantages of conventional IIF reading led to development of a software tool for automated image analysis. Without the requirement for additional high-cost instrumental equipment the *Fluorolyzer* software provides single-cell fluorescence quantification on digital images from IIF assay microscopy (Manuscript III). The experimental setup enables the operator to visually check antibody bound structures and to exclude manually defective cells or artifacts from measurement. Based on fluorescence data serum titers of antigen-specific antibodies can be calculated. This approach improves the conventional IIF procedure by providing objective, quantitative, and re-analyzable fluorescence data. Moreover, the authors reported significant correlation of software-based titer results to manually determined titers reflecting the reliability of automated image analysis. The here presented combination of standard IIF assays with software-based fluorescence quantification and titer calculation may provide a basic analytical platform for further automatization. Also current commercial IIF systems are directed towards fully automated, multiplexed immunofluorescence microscopy including slide reading and serological interpretation. The InoDiag system (France) provides microarrays for serological detection of antibodies against multiple pathogens causing atypical pneumonia or infectious endocarditis (Gouriet et al. 2008 (a); Gouriet et al. 2008 (b)), whereas the slide-based EUROPattern system (Germany) comprises autoimmune disease serology with automated detection of anti-nuclear antibodies and anti-dsDNA antibodies (Gerlach et al. 2015; Gorgi et al. 2015). Besides antibody testing to antigens from pure bacterial cultures further applications for *Fluorolyzer* software might possible. As this tool is

not restricted to fixed fluorescence staining combinations it is conceivable to combine different immunofluorescence stainings or DNA stainings as well as FISH (Lübeck, Hansen and Sørensen 2000). In this context the manual cell selection mode of *Fluorolyzer* also would allow targeted analysis of a single cell type in a mixture of species or in complex environmental samples enabling even detection of antibodies against non-cultivable species.

Analysis of seroprevalences of specific antibodies in occupationally exposed persons provides the possibility to identify associations of potential immunogenic properties of single microbial species to work-related symptoms and disorders. Furthermore, monitoring of specific antibodies is an essential method to diagnose current or past infections and provides valuable information about infection risks in epidemiological analyses. After initial antigen contact high levels of IgM are detectable. Later during the course of the immunoreaction IgM production declines to baseline levels and is substituted by IgG release (Hof and Dörries 2005). Although exposure in poultry processing is characterized by occurrence of multiple pathogenic species and a broad range of health effects, only few studies aimed at determination of anti-microbial antibodies in sera from poultry workers. For example, specific antibody titers to *Campylobacter* spp. are suitable markers for serological investigation of campylobacteriosis (Janssen et al. 2008) and a recently published study reported significantly higher seroprevalences of *Campylobacter*-specific antibodies in an exposed cohort of duck farm workers than in a control cohort (Masanta et al. 2016). Likewise, Skórska et al. (2007) investigated work-related health effects among chicken hatchery workers. Analysis of serum precipitin reactions to twelve different bacterial and fungal antigens revealed a higher seroprevalence of specific antibodies against *E. coli* and *Acinetobacter baumannii*. Following this approach successful establishment of *Fluorolyzer* software allowed its employment for determination of duck hatchery workers' serum titers to airborne bacteria (Manuscript IV). The set of tested isolates comprised eleven different isolates from eight species and represents the predominant bacteria groups in duck hatchery air: *Enterococcus* spp., *Staphylococcus* spp., *Acinetobacter* spp. and *Enterobacteriacae* (Martin and Jäckel 2011). Since for most isolates no substantial titer differences between the reference group and the exposed worker group were detected, entire titer data were employed for cluster analysis to detect potential grouping. This new approach revealed that immunoreaction patterns from workers did not generally differ from those of control persons after long term bioaerosol exposure. These results were further confirmed by a new assay strategy utilizing the diversity of microbial cells in complex bioaerosol samples as antigens for indirect immunofluorescence testing. Complete bioaerosol samples also include eukaryotic cells of fungi or protozoa reflecting the

entire proportional microbial exposure at specific workplaces. Hence, the bioaerosol assay covers the overall immunoreactivity on complex workplace samples and provides a comprehensive detection of anti-microbial antibodies in human sera. Although results from both IIF test systems, either with pure cultures or with bioaerosol samples exhibited a large conformity of antibody profiles for exposed and non-exposed persons, workers' titer results for a few single isolates are still remarkable. Next to a significantly increased mean workers' titer against an isolate of *Proteus mirabilis*, in particular higher mean titers to three of four distinct *A. baumannii* isolates attracted attention. These results support the findings of Skórska et al. (2007) when they reported significant more precipitin reactions to *A. baumannii* antigens in a group of chicken hatchery workers than in the reference group. However, they did not detect a correlation to the prevalence of work-related symptoms. Relevance of *A. baumannii* as a pathogen is defined by frequent appearance as causative agent of nosocomial infections including meningitis, bacteremia and pneumonia (Peleg, Seifert and Paterson 2008) as well as by the global spread of multidrug-resistant strains (Zarrilli et al. 2013). Albeit PCR-based typing of the isolates revealed no affiliation to the successful and mainly hospital-associated international clones 1-3, further investigations regarding potential involvement in pathogenesis of occupational respiratory disorders are required.

When using the aforementioned bioaerosol assay identity of individual fluorescence labelled cells is unknown. For this reason separation and subsequent identification of single antibody fluorescent cells would close a gap in comprehension of immunogenic species in agricultural environments and would facilitate specific investigations concerning bacterial involvement in work related respiratory disorders. Here promising Laser Microdissection and Pressure Catapulting (LMPC) technology provides a high degree of certainty for selecting exclusively target cells from bioaerosol samples. This is a key issue for a reliable analysis. Next to characterization of eukaryotic cells or tissues in plant physiology (Hogekamp et al. 2011), forensics (Di Martino et al. 2004) or pathology (Lehmann et al. 2000) LMPC also has been used for analysis of particular bacteria in complex samples. In order to distinguish target cells from non-target cells during LMPC procedure phenotypic or genotypic characteristics can be employed. A common method to visualize specific bacterial cells in complex samples is FISH (Klitgaard et al. 2005; Smith et al. 2011). In contrast, Gloess et al. (2008) enabled phylogenetic characterization of poly-phosphate accumulating bacteria by applying DAPI for labelling. A novel approach aimed at combining IIF and LMPC technology for separation of immunogenic bacterial cells from complex workplace bioaerosols (Manuscript V). While solutions for technical key issues as antibody specifity, applicability of base materials and

DNA extraction from minimal cell numbers have been established successfully, further efforts for a reliable 16S rRNA gene analysis of LMPC separated bacteria will be necessary.

1.5 Conclusions and Prospects

In contrast to other agricultural production facilities air quality in hatcheries is rarely characterized. However, according to current German legislation employers shall assess potential health risks associated to work. This includes health risks from biological agents, although a measurement of the actual microbial burden is not mandatory. The results presented in this thesis provide novel data in regard to microbial air contamination in hatcheries and may support establishment of specific risk assessments. In the air of the investigated chicken and turkey hatcheries high concentrations of bacteria were detected, in particular during tasks with contact to contaminated poults and eggshells. Albeit diversity was substantially lower than in poultry houses, bacterial communities in hatcheries displayed an extensive similarity on the genus level indicating the presence of similar ecological conditions in both facilities independently from the poultry species. Based on 16S rRNA gene analysis several species are most closely related to prominent risk group 2 pathogens e.g. *E. faecalis*, *S. aureus* or *A. baumannii*. However, potential health risks associated to these species are mostly derived from clinical cases of infection and do not necessarily reflect their relevance in occupational settings. For this reason future investigations shall investigate the role of workplace species in infection of workers' respiratory tract. For example, *Acinetobacter* species are known to colonize human skin and throat (Seifert et al. 1997; Chu et al. 1999; Anstey et al. 2002) but evidence for occupationally acquired infections has not been provided so far. In this context respiratory disorders among agricultural workers are likely to be underdiagnosed as it might be difficult to associate unspecific symptoms with occupational bioaerosol exposure.

Determination of specific antibody responses by immunofluorescence based techniques as described in this thesis appeared to be useful tools for characterization of workers' immunoreactions after long term bioaerosol exposure. While image-based titer determination with single isolates enables identification of immunogenic workplace species with an improved accuracy in comparison to conventional readout, the bioaerosol assay allows evaluation of antibody responses to complex samples. For separation and identification of antibody labelled microbial cells in such bioaerosol samples LMPC is a promising technique, although further development efforts are necessary.

Characterization of poultry bioaerosols by different approaches revealed substantial differences between cultivation dependent quantification and total cell counts as well as between cultivation dependent identification and the molecular cloning approach. These findings emphasize the need for a further advancement in standardization of sampling and sample processing. Limitations of cultivation dependent quantification of specific or total bacteria can be addressed by molecular methods. As an alternative quantitative PCR technology next to qRT PCR, that can be affected by inhibitors present in environmental samples (Stults et al. 2001), recently droplet digital PCR (ddPCR) has been introduced. This approach enables the quantification of absolute target concentrations without the need for external calibrators (Pinheiro et al. 2011; Hindson et al. 2011) and seems to be a more sensitive tool for analysis of target DNA in complex samples (Kim, Jeong and Cho 2014). Additional efforts in covering microbial exposure shall focus on the tenacity of airborne microorganisms and the differentiation of viable and non-viable as these are crucial criteria for risk assessment (Tang 2009; Cangelosi and Meschke 2014). While non-viable microorganisms are not infectious anymore they might still have toxic or allergenic potential. Cultivation based approaches or total cell count provide only limited information on the physiology of microorganisms. These limitations could be addressed by implementation of strategies as live/dead staining using propidium iodide (Davey 2011) or viability PCR using membrane-impermeative reagents such as propidium monoazide (PMA) in exposure characterization (Nocker and Camper 2009).

Next to health risk arising from occupational exposure to bacteria and fungi, health hazards may arise also from airborne viruses. However, the airborne viral community at agricultural workplaces is even less characterized than for bacteria and fungi, most likely due to the technical challenges related to detection and identification (Prussin, Marr and Bibby 2014). Bacteria share 16S rRNA genomic marker regions and fungi have conserved ribosomal internal transcribed spacer (ITS) regions that can serve as universal markers in community analysis (O'Brien et al. 2005; Schoch et al. 2012). In contrast, for viruses a common genetic element is unknown (Rohwer and Edwards 2002). For this reason metagenome analyses have been used to examine the composition of viral communities, e.g. in marine (Angly et al. 2006), soil (Fierer et al. 2007) and sludge environments (Bibby and Peccia 2013). So far this approach has not been applied for investigation of agricultural bioaerosols. However, seroprevalence studies among swine and poultry workers revealed high antibody titers against avian and swine influenza viruses indicating elevated risks for viral zoonotic infections (Swayne 2005; Myers et al. 2006; Gray and Baker 2007; Wang et al. 2014).

Due to the complexity of bioaerosol-host interactions future research shall include comprehensive approaches for diagnosis of work-related respiratory disorders. Two recently published high-impact studies present strategies that might serve as pioneering examples. Xu and colleagues (2015) combined DNA microarray synthesis and bacteriophage display to create a synthetic representation of peptide epitopes comprising the human virome (VirScan). While current serological methods are limited to test one pathogen at a time this new technique could simultaneously detect responses to all human viruses (> 1000 strains from 206 species). This method enables profound analysis of viral exposure in a large number of individuals and can be expanded also for peptides of bacteria, fungi, and protozoa. A second promising approach presented by Tsalik et al. (2016) also utilizes powerful microarray technology. Analysis of gene expression patterns in peripheral whole blood allowed reliable discrimination of non-infectious from infectious illness and bacterial from viral causes of acute respiratory infection. To conclude, application of innovative high-throughput techniques such as serological profiling and host response classifiers creates an opportunity for more in-depth characterization of health effects caused by bioaerosol inhalation.

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2 MATERIAL AND METHODS

Material and Methods used for this doctoral thesis are documented in the listed manuscripts:

Method	Manuscript
Characterization of Microbial Exposure in Poultry Hatcheries	
Air sampling	I, II
Eggshell sampling	II
Cultivation dependent quantification and isolation of bacteria and fungi	I, II
Total cell count	I, II
Cell lysis, DNA extraction	I, II
PCR and 16S rRNA gene analysis	I, II
Clone library construction	I, II
Estimation of bacterial diversity	I, II
Restriction fragment length polymorphism (RFLP) analysis	II
Detection of Workers' Antibody Responses to Airborne Bacteria	
Indirect immunofluorescence assays	III, IV
Titer determination by eye	III
Image-based fluorescence quantification	III
Titer calculation and statistical analyses	III, IV
Cluster analysis	IV
Strain typing of *Acinetobacter baumannii* isolates	IV
Proportions of antibody-bound microbial cells in bioaerosol samples (bioaerosol assay)	IV
Laser Microdissection and Pressure Catapulting (LMPC)	V
Fluorescence staining for LMPC	V
Cell lysis and DNA extraction after LMPC	V
PCR after LMPC	V
Multiple displacement amplification	V

3 SCIENTIFIC MANUSCRIPTS

3.1 Characterization of Microbial Exposure in Poultry Hatcheries

Manuscript I

Occupational Exposure to Airborne Bacteria in Different Poultry Hatcheries

unpublished

Occupational Exposure to Airborne Bacteria in Different Poultry Hatcheries

Abstract

In current large-scale poultry production employees are exposed to high concentrations of airborne microorganisms. Although long term inhalation of agricultural bioaerosols is related to an increased risk of respiratory disorders, microbial communities in workplace air and their contribution to pathogenesis are rarely characterized. In this study workers' microbial exposure in a chicken and a turkey hatchery was investigated by a combination of conventional cultivation based techniques and molecular biological methods. During different tasks with handling of poults or eggshells highest concentrations of mesophilic airborne bacteria were detected in the chicken hatchery both by colony count (5.4×10^5 cfu m^{-3}) and total cell count (5.3×10^6 cells m^{-3}). Levels in the turkey hatchery were lower, gaining 1.4×10^4 cfu m^{-3} and 1.4×10^6 cells m^{-3}. In addition, airborne moulds were quantified in both hatcheries albeit concentrations (2.6×10^2 - 6.2×10^2 cfu m^{-3}) were comparable to those in outside air. Analysis of bacterial community compositions by construction of clone libraries displayed a low species diversity but an extensive similarity on the genus level in both hatcheries, revealing *Enterococcus*, *Clostridium*, *Staphylococcus* and *Acinetobacter* as predominant genera. Moreover, identification of bacterial and fungal isolates confirmed occurrence of various pathogens which are classified as risk group 2 microorganisms (German TRBA 460, 466). In conclusion, processing of chicken and turkey poults is accompanied by high concentrations of potentially harmful airborne microorganisms. Independently of the poultry species, hatchery workers are confronted to a mostly similar microbial exposure. Furthermore, hatchery bioaerosols comprise a specific microbial composition and display substantial differences to bioaerosols in poultry houses.

Introduction

Since methods for large-scale poultry and livestock production moved to high stocking densities and a trend to full-time employment has developed, workers face an increased risk for respiratory disorders. Occurrence of chronic pulmonary disease, hypersensitive pneumonitis, increased airway responsiveness and decline in lung function [1-5] among poultry farm workers focused research on agricultural bioaerosols. These are complex mixtures of organic air pollutants like gases, dust, endotoxins as well as bacteria, fungi and viruses [1, 6]. Nevertheless, microbial communities in workplace bioaerosols are rarely characterized and their contribution to pathogenesis of respiratory diseases is not well understood. As a consequence establishment of an adequate risk assessment is difficult and characterization of occupational exposure is limited by the absence of standardized protocols for microbial quantification and identification. Although alternative molecular biological methods as fluorescent cell counting [7] or quantitative Real-Time PCR (qRT PCR) [8, 9] are available for microbial quantification, classic cultivation and colony counting is still commonly used. However, cultivation based techniques generally suffer from major drawbacks when aiming at quantification of microbial air contamination. Unknown demands for cultivation conditions of many species as well as desiccation stress leading to death or "viable-but-not-culturable" (VBNC) state [10] during sampling may cause significant underestimation of actual levels of airborne bacteria [11, 12]. Next to underestimation non-detection of species is a serious problem of cultivation based identification. This problem is addressed by PCR based methods that avoid time-consuming isolation and enable specific and sensitive detection by targeting bacterial genome sequences in environmental samples [13, 14]. In particular, analysis of variable regions in highly conserved 16S rRNA genes allows taxonomic classification of prokaryotes [15]. In combination with molecular cloning this technique has been successfully applied for screening and quantification of rRNA gene sequences in workplace bioaerosols [7, 16], thereby enabling new insights into bacterial community composition.

In contrast to poultry sheds, hatcheries appear clean due to absence of feed, litter and feces. Therefore, possible sources of bioaersols are less obvious, in particular since hatching eggs are subject of intensive sanitization and incubators are cleaned and disinfected after each hatching cycle. Conceivably in consequence of this uncontaminated appearance only few studies report on workers' particular microbial exposure in poultry hatcheries. Martin et al. [17] reported high concentrations of airborne bacteria in a duck hatchery during processing of newly hatched ducklings and detected a corresponding decline in lung function in hatchery

workers. In addition, Skorska et al. [18] investigated workers' immunological parameters in a chicken hatchery and found higher total immunoglobulin E (IgE) concentrations compared to a control group. These results may indicate potential harmful impacts of hatchery bioaerosols in an allegedly unpolluted work environment and underline the need for further investigations in exposure and effect characterization.

The aim of this study was to investigate workers' exposure to bioaerosols in a chicken and a turkey hatchery. By using a combination of conventional cultivation dependent methods as well as molecular biological methods, the analyses focused on the impact of different poultry species on microbial community composition and comparison of levels of airborne microorganisms during various work activities.

Material and Methods

Work Activities in Hatcheries

In the investigated German chicken and turkey hatchery poults are mainly hatched for fattening in commercial farms. The hatchery's weekly work schedule is defined by days with regular removal of poults from hatcher incubators (up to 3 per week) and days without poult removal. At days with regular removal several ten thousands of poults are processed by 10 to 15 workers for the entire work shift and the following typical work activities are performed: "Sorting" means the manual separation of healthy poults from eggshells, dead or inadequate animals and is the initial task after removal of the poults from hatcher incubators. "Sexing" is the determination of a poult's sex by visual inspection of the vent and happens after sorting. "Debeaking" is performed after sexing and means the manual transfer of chicken and turkey poults to automated or non-automated debeaking systems. "Vaccination" means application of vaccine to the poults by injection or spraying. "Waste shredding" is performed by transferring eggshell waste as well as dead or inadequate animals to a special waste chopper. Final tasks include manual high-pressure cleaning and disinfection of equipment, incubators and work areas as well as preparation of the poults for shipping. In contrast, at days without poult removal tasks do not include any poult handling: "Candling" refers to the screening of eggs with a bright light source for monitoring the development of the embryo. "Egg transfer" refers to the manual transfer of eggs from setter incubators to hatcher incubators. "Deposition of wood wool in transport baskets" means the manual loading of transport baskets with wood wool for safe shipping of the poults. In addition, routine maintenance procedures are carried out.

Air Sampling

Airborne microorganisms were collected during different work activities at days with regular removal of poults from hatcher incubators and at days without poult removal. In order to generate reference values for indoor exposure, outside air was collected with stationary and personal sampling systems on the windward side of the hatcheries. For stationary sampling MD8 filtration devices (Sartorius, Germany) were used. Sampling was performed at a height of 1.5 m in close proximity to active workers with a flow rate of approx. 1.8 m^3 h^{-1} (MP2/39, Holbach GmbH, Germany). The exact sampled air volume was monitored by calibrated gas meters (Remus 4; GSA, Germany) and the mean flow velocity at the active filter site (Ø 7 cm) was 0.12 m s^{-1}. For total cell count and generation of clone libraries bioaerosols were collected on polycarbonate filters (Ø 76 mm, 0.8 µm pore size; Whatman, Germany). For cultivation airborne microorganisms were collected on gelatin filters (Ø 78 mm, 3.0 µm pore size; Whatman, Germany) and for each working activity three bioaersol samples were collected sequentially (each 10 min according to 3 x 0.3 m^3). Workers' individual exposure was gathered with personal air sampling pumps (GilAir Plus, Sensidyne; USA). Pumps were operated with a flow rate of 3.5 l m^{-1} and airborne microorganisms were collected on polycarbonate filters (Ø 37 mm, 0.8 µm pore size; Whatman, Germany). Cells collected on polycarbonate filters were detached from filter surface into 10 ml cell free NaCl solution (0.9%) using a Stomacher (Stomacher 80 Biomaster, Seward, UK) at maximum agitation for 1 min.

Total Cell Count

For total 4',6-diamidino-2-phenylindole (DAPI)-fluorescence cell count [19, 20], microbial cells in 5 ml of bioaerosol suspensions were fixed and stabilized by addition of 0.5 ml formaldehyde [37 % (v/v)]. After a minimum storage time of 24 h at 4°C, 10 µl of DAPI solution (1 mg ml^{-1}) were added to 1 ml of the fixed cells. After 30 min of incubation in darkness, the stained cells were quantitatively filtrated on a black polycarbonate filter (0.2 µm pore size, Ø 25 mm; Millipore, Germany) using a filtration unit (Ø 20 mm, 30 ml; Sartorius, Germany). Filters were embedded in an anti-fading reagent (AF1, Citifluor Ltd; UK). Fluorescence-labelled cells were counted with an epifluorescence microscope (Leica, DMIRB) at 1000-fold magnification equipped with a counting ocular.

Cultivation Analyses

Three loaded gelatin filters with bioaerosol samples were combined and dissolved in 10 ml NaCl solution at 40°C. For quantification and isolation, serial dilutions were plated in triplicate on non-selective casein-soy agar (Carl Roth, Germany), CATC agar (citrate azide tween carbonate) for enterococci, MacConkey agar (Oxoid, UK) for *Enterobacteriaceae*, CHROMagar for *Staphylococcus aureus* (CHROMagar, France), CHROMagar for *Acinetobacter (*CHROMagar, France), dichloran-glycerol agar (Carl Roth, Germany) and Sabouraud-dextrose agar (Oxoid, UK) for moulds and yeasts and incubated at 37°C or 26°C (casein-soy agar) for 7 days. Bacterial isolates were characterized according to cell and colony morphologies and Gram staining. Isolate concentrations were estimated by counting colonies of identical morphology. Mould isolates were identified microscopically (Landesgesundheitsamt Baden-Württemberg or Umweltmykologie Dr. Dill und Dr. Trautmann GbR) and one yeast isolate was identified with a Vitek 2 Compact system (bioMérieux, France).

16S rRNA Gene Analysis

Genomic DNA extraction from isolates, 16S rRNA gene amplification, sequencing and sequence analysis was performed according to Martin, Kämpfer and Jäckel [20]. In brief, a loop of bacterial cells was transferred and mixed with 0.5 g zirconia beads (0.1 mm, Carl Roth; Germany) in a reaction tube (1.5 ml). Afterwards cells were disrupted by shaking this mixture on a vortexer (Vortex-Genie 2; Carl Roth, Germany) for 2 min. Subsequent DNA extraction steps were performed with the GenElute Plant Genomic DNA Miniprep Kit (Sigma Aldrich, USA) following the instructions of the manufacturer. The 16S rRNA genes were partially amplified (fD1 and rP1 primer) [21] and amplicons were purified with Qiaquick PCR purification Kit (Qiagen, Germany). 16S rRNA gene sequences were processed with SeqTrace software package [22] and assigned by comparison with provided sequences of bacterial type strains in GenBank (sequence similarity of ≥ 98 %). Phylogenetic trees were calculated and generated using the neighbor-joining method supplied by the MEGA 5.0 package [23, 24].

Clone Libraries

Generation of clone libraries was performed according to Martin and Jäckel [16]. Cells from the remaining 5 ml bioaerosol suspensions were concentrated to pellets by centrifugation (21.380 x g, 15 min) and were used for DNA extraction and subsequent amplification of 16S rRNA genes as described above. Amplicons of 16S rRNA gene sequences were employed to generate *E. coli* clone libraries (pGEM®-T vector system kit; Promega, USA). 300 clones were randomly picked from each clone library. Plasmid inserts were sequenced (approx. 1350 bp) using fD1 and rP1 primer. Cloning and sequencing was performed by LGC Genomics (Berlin, Germany). Phylogenetic assignments were performed as described above. Operational taxonomic units (OTU) were defined as single clones or groups of sequences having 98 % or more similarity.

Estimation of Bacterial Diversity

A rarefaction curve was determined with Analytic Rarefaction 1.3 (http://strata.uga.edu/software) [25-27]. The coverage described by Good [28] estimates the proportion of phylotypes in a library of infinite size that would be represented in a smaller library:

$$C = 1 - \frac{n_1}{N}$$

n_1 is the number of phylotypes appearing only once in a library and N is the library size [29]. In order to estimate the probable total number of phylotypes, the S_{Chao1} estimator was applied [29]:

$$S_{Chao1} = S_{obs} + \frac{F_1^2}{2(F_2 + 1)} - \frac{F_1 F_2}{2(F_2 + 1)^2}$$

S_{obs} is the number of phylotypes observed in the library and F_1 and F_2 are the number of phylotypes occurring either one or two times.

Results

Concentrations of Airborne Microorganisms

Concentrations of airborne microorganisms in a chicken and a turkey hatchery were detected by cultivation analyses and total cell count. Exposure during different work activities with poult handling at a day with regular removal of poults from hatcher incubators was compared to exposure at a further day without poult removal and to outside air. Highest bacterial concentrations were detected on casein-soy agar. In the chicken hatchery concentrations of mesophilic airborne bacteria during sorting, debeaking and vaccination displayed similar levels between 2.2 x 10^5 and 5.4 x 10^5 cfu m^{-3} (Fig. 1). In contrast, mean bacterial concentrations in outside air and during activities without poults were substantial lower with 9.8 x 10^2 and 1.8 x 10^3 cfu m^{-3}, respectively. Furthermore, the mean concentration of moulds in chicken hatchery air when handling poults (2.6 x 10^2 cfu m^{-3}) displayed a level comparable to that of outside air (1.7 x 10^2 cfu m^{-3}) but no moulds were detected during activities without poults. Although concentrations of airborne bacteria were lower than in the investigated chicken hatchery, levels in the turkey hatchery were also similar for different tasks with poult handling (8.4 x 10^3 - 1.4 x 10^4 cfu m^{-3}). The mean concentrations of bacteria in outside air and during activities without poults were 2.4 x 10^3 cfu m^{-3} and 4.5 x 10^1 cfu m^{-3}, respectively. Moreover, mean concentrations of airborne moulds were similar in outside air (4.8 x 10^2 cfu m^{-3}) and for activities with and without poults ranging from 3.9 x 10^2 to 6.2 x 10^2 cfu m^{-3}.

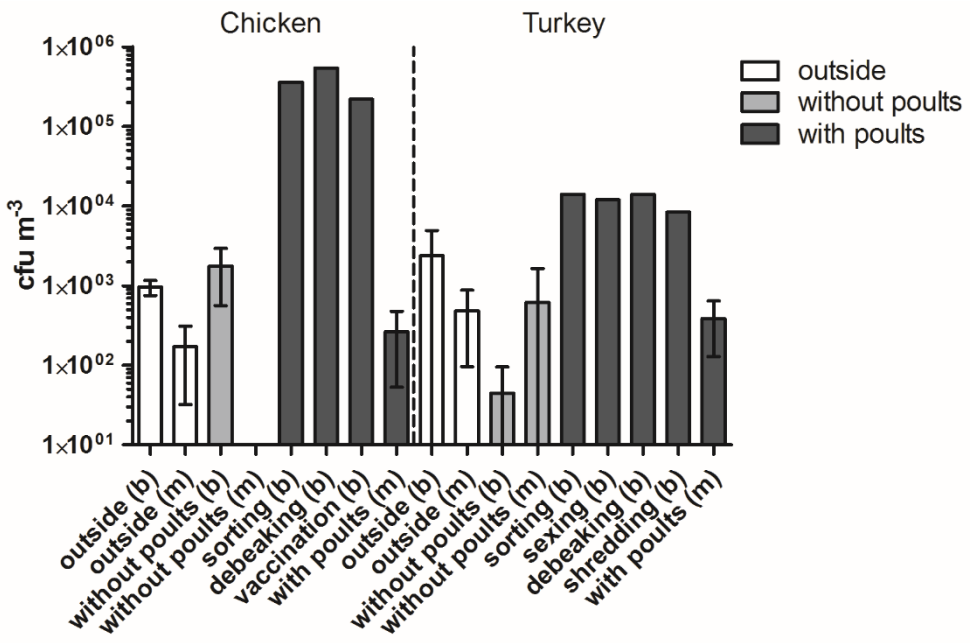

Figure 1 Mean colony forming units per m^{-3} with standard deviations of bacteria (b) on casein-soy agar and moulds (m) on dichloran-glycerol agar or casein-soy agar from various working activities (stationary sampling) with and without poult handling in a chicken and a turkey hatchery.

Results from total cell counts displayed similar exposure patterns as results from the cultivation approach. Mean concentrations of airborne microorganisms in chicken hatchery air during activities with poult handling were similar for sorting/debeaking (5.3 x 10^6 cells m^{-3}) and vaccination (3.3 x 10^6 cells m^{-3}) (Fig. 2). In contrast, the mean concentration for tasks without poults was lower by two orders of magnitude (5.2 x 10^4 cells m^{-3}) and the mean concentration in outside air was 5.5 x 10^5 cells m^{-3}. Different work activities with turkey poults displayed similar mean concentrations of airborne microorganisms (5.1 x 10^5 - 1.4 x 10^6 cells m^{-3}) but levels were lower than in the chicken hatchery. Compared to levels of work activities with poults, tasks without poults (1.1 x 10^5 cells m^{-3}) and outside air (3.9 x 10^4 cells m^{-3}) displayed lower concentrations of airborne microorganisms. Comparing the results from total cell count and colony count in both hatcheries, values for colony forming units were with varying extent substantial lower than values for microbial cells.

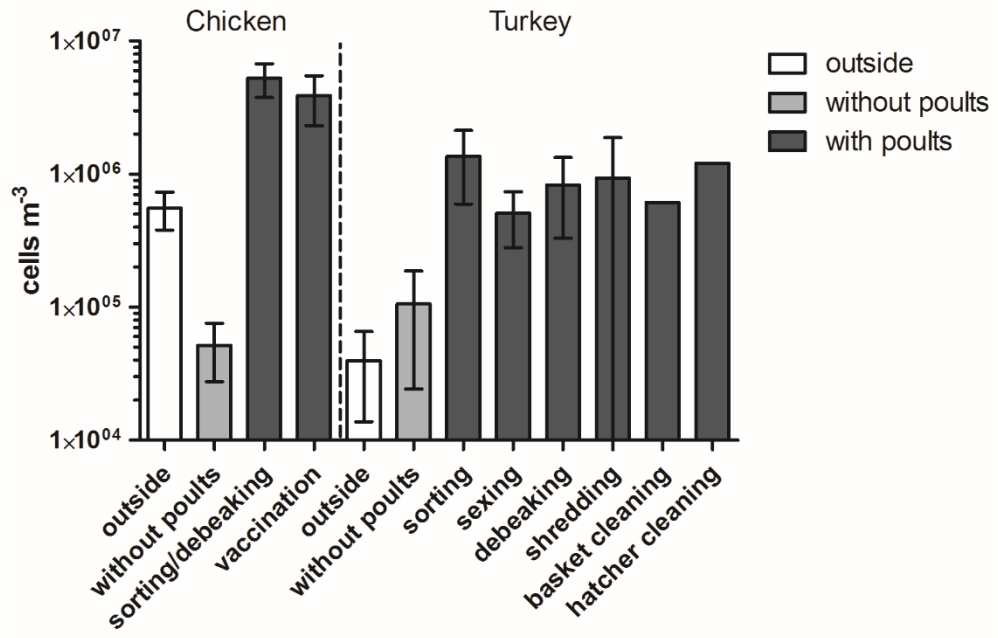

Figure 2 Mean total cell counts per m^{-3} with standard deviations from various working activities (stationary and personal sampling) with and without poult handling in a chicken and a turkey hatchery.

Microbial Communities in Hatchery Air

Analysis of the composition of bacterial communities in hatchery air during poult sorting was performed by generation of 16S rRNA gene clone libraries. Furthermore, bacterial and fungal isolates were identified. Combined methods revealed a total of 18 bacterial genera and 29 species in the chicken hatchery as well as 20 genera and 33 species in the turkey hatchery. On the genus level bacterial community compositions in the chicken and turkey hatchery display a broad similarity (Fig. 3). Accounting for more than 80 % of the clones, abundant operational taxonomic units (OTUs) of clone libraries from both hatcheries were most closely related to 16S rRNA gene sequences of the genera *Enterococcus, Clostridium, Staphylococcus* and to a lesser extent *Acinetobacter*. The estimated total number of phylotypes was 74 for both clone libraries and Good's coverage for the chicken and turkey hatchery clone library was 94 % and 89 % respectively. In combination with the rarefaction analysis (Fig. 4) these parameters indicated that the identified 16S rRNA gene sequences most likely represent the majority of bacterial sequences present in hatchery bioaerosol samples. 16S clone library analysis of the bacterial community composition in chicken hatchery air on the species level revealed next to predominant *Clostridium* spp. (29.7 %) major occurrence of *Enterococcus faecalis* (19.1 %), *Staphylococcus aureus* (10.3 %), *Enterococcus faecium* (9.4 %) and *Acinetobacter baumannii* (7.3 %) (Tab. 1a). Using different culture media for cultivation 34 bacterial isolates were recovered from chicken hatchery air. Abundant isolates were most closely related to *E. faecalis* (9×10^4 cfu m^{-3}), *Staphylococcus agnetis/hyicus* (6×10^4 cfu m^{-3}) as well as *A. baumannii* (4×10^4 cfu m^{-3}) (Tab. 1a). Further isolates were assigned to the genera *Staphylococcus, Bacillus, Escherichia, Kocuria, Leclercia, Nocardia, Pantoea, Sphingomonas* and *Stenotrophomonas*. Although analysis of 16S clone libraries from chicken and turkey hatchery air displayed extensive similarity on the genus level, minor differences were detected on the species level. Next to *Clostridium* spp. (34.9 %) dominating species in turkey hatchery air were *E. faecalis* (25.8 %), *Staphylococcus lentus* (7.0 %) and *Enterococcus lactis* (5.9 %) (Tab. 1b). Furthermore, a total of 58 bacterial isolates was obtained from cultivation and abundant isolates were most closely related to *E. faecalis* (9×10^3 cfu m^{-3}) as well as *Bacillus* spp. (4×10^3 cfu m^{-3}) (Tab. 1b). Further isolated species were assigned to the genera *Staphylococcus, Bacillus, Brevibacterium, Chryseobacterium, Enterobacter, Klebsiella, Pseudomonas, Citrobacter, Comamonas, Dermacoccus, Escherichia, Lysinibacillus, Macrococcus* as well as *Microbacterium*. In addition to bacterial isolates also fungal isolates were recovered from workplace air in both hatcheries (1×10^2 - 3×10^3 cfu m^{-3}). In both hatcheries *Aspergillus fumigatus* and *Trichoderma* sp. were detected.

Further isolates from chicken hatchery were identified as *Aspergillus sydowii*, *Aspergillus versicolor*, *Penicillium* sp., *Penicillium chrysogenum* and *Scopulariopsis brevicaulis* (Tab. 1a) whereas in the turkey hatchery *Candida* sp., *Trichoderma longibrachiatum* and *Trichoderma koningii* were found additionally (Tab. 1b).

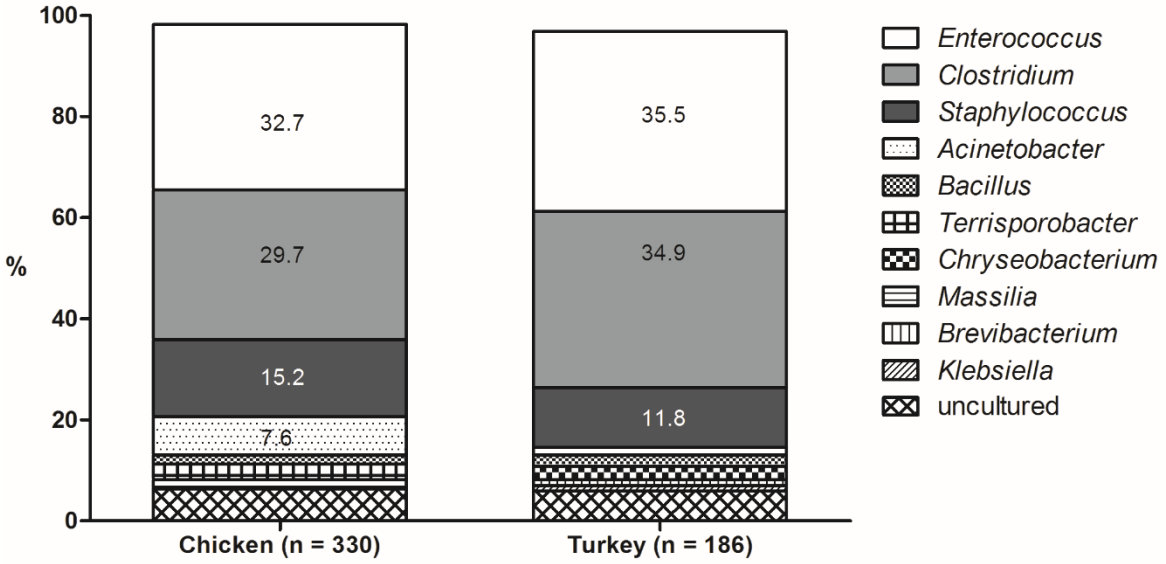

Figure 3 Percental composition of genera (> 1%) in 16S rRNA clone libraries (~ 1350 bp sequence length) generated from airborne bacteria in hatchery air during poult sorting.

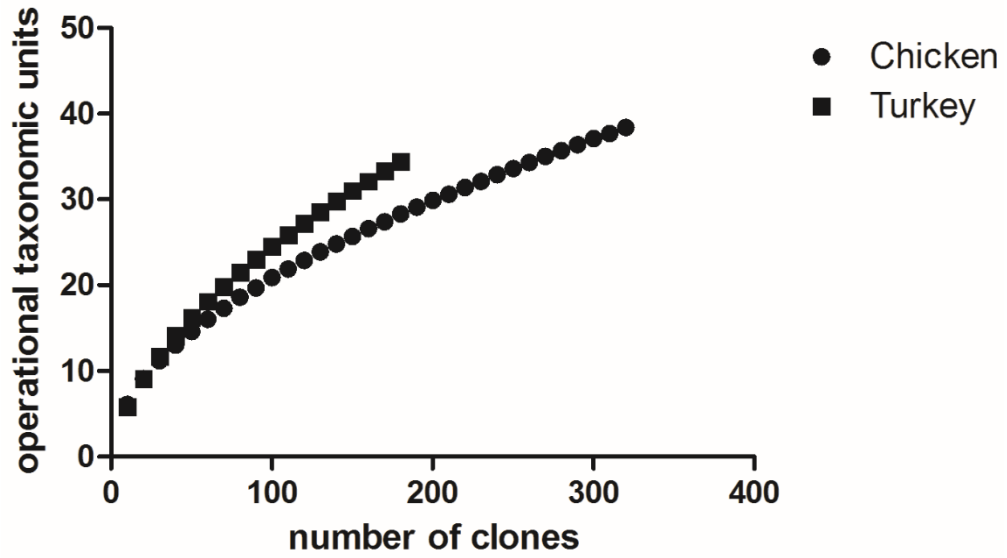

Figure 4 Rarefaction analyses of 16S rRNA gene clone libraries generated from airborne bacteria in hatchery air during poult sorting. The rarefaction curve plots the number of observed operational taxonomic units as a function of the number of clones.

Table 1 Identification of 16S rRNA gene sequences (~ 1350 bp) obtained either from isolates or from clone libraries of investigated chicken (a) and turkey hatcheries (b). Left columns display most closely related type strains or genera based on 16S rRNA gene sequence comparison and the corresponding risk group (German Technical rules for Biological Agents, TRBA 466). For isolates obtained from cultivation, sequence similarity (%) and estimated concentrations are given. For clone sequences, clone number, sequence similarity (%) and relative frequency (Rel. freq.) (%) are given. (n. d., not determined)

a) Chicken Hatchery			Cultivation		Clone Library		
	Identification	Risk group	Sequence similarity	Est. cfu per m^{-3}	Clone number	Sequence similarity	Rel. freq.
Both methods	*Acinetobacter baumannii*	2	100	4×10^4	24	99.5-100	7.3
	Bacillus sp.	n. d.	100	3×10^4	6	98.9-100	1.8
	Enterococcus faecalis	2	99.8–100	9×10^4	63	99.2-100	19.1
	Staphylococcus agnetis/hyicus	n. d./2	98.9-100	6×10^4	12	99.2-100	3.6
	Staphylococcus aureus	2	99.1-100	1×10^4	34	99.2-100	10.3
	Staphylococcus chromogenes	2	98.9-100	5×10^3	4	99.2–99.7	1.2
Cultivation	*Escherichia fergusonii*	2	99.6	1×10^2			
	Kocuria rhizophila	1	99.3	1×10^4			
	Leclercia adecarboxylata	2	98.9	2×10^2			
	Nocardia globerula	1	100	1×10^4			
	Pantoea eucalypti	n. d.	99.8	1×10^2			
	Sphingomonas panni	1	99.8	2×10^4			
	Stenotrophomonas maltophilia	2	99.8	1×10^2			
	Stenotrophomonas rhizophila	1	100	1×10^4			
Clone Library	*Acinetobacter lwoffi*	2			1	99.5	0.3
	Brevundimonas faecalis	n. d.			1	99.3	0.3
	Brevundimonas subvibrioides	1			1	98.6	0.3
	Chryseobacterium bernadetti	n. d.			1	99.4	0.3
	Clostridium sp.	n. d.			98	98.1-100	29.7
	Enterococcus durans	2			3	99.8	0.9
	Enterococcus faecium	2			31	98.1–99.7	9.4
	Enterococcus hirae	2			3	99.5-100	0.9
	Enterococcus lactis	n. d.			8	99.5–99.7	2.4
	Massilia aurea	1			3	98.3–99.5	0.9
	Massilia plicata	1			1	99	0.3
	Massilia timonae	1			2	98.4	0.6
	Psychrobacter namhaensis	1			1	99.3	0.3
	Shigella dysenteriae	2/3**			1	99.3	0.3
	Terrisporobacter mayombei	n. d.			10	98.7–99.1	3.0
	uncultured				22	< 98	6.7
Fungi	*Aspergillus fumigatus*	2		1×10^2			
	Aspergillus sydowii	n. d.		1×10^2			
	Aspergillus versicolor	1		2×10^2			
	Penicillium chrysogenum	n. d.		1×10^2			
	Penicillium sp.	n. d.		1×10^2			
	Scopulariopsis brevicaulis	1		1×10^2			
	Trichoderma sp.	n. d.		1×10^2			

b) Turkey Hatchery			Cultivation		Clone Library		
	Identification	Risk group	Sequence similarity	Est. cfu per m^{-3}	Clone number	Sequence similarity	Rel. freq.
Both methods	*Bacillus* sp.	n. d.	99.8	4×10^3	4	99.6–99.7	2.2
	Brevibacterium luteolum	1	99.8	1×10^3	2	99.8	1.1
	Chryseobacterium gleum	2	99.9	2×10^3	5	98.4–99.8	2.7
	Enterobacter asburiae/xiangfangensis	2/n. d.	100	2×10^3	1	99.7	0.5
	Enterococcus faecalis	2	99.9-100	9×10^3	48	98.3-100	25.8
	Klebsiella pneum./quasipneum.	2/n. d.	99.7–99.9	1×10^3	2	99.2–99.3	1.1
	Pseudomonas guariconensis	n. d.	99.9-100	1×10^3	1	99.9	0.5
	Staphylococcus lentus	1	98.5-100	1×10^3	13	99.6-100	7.0
Cultivation	*Bacillus kokeshiiformis/thermolactis*	n. d.	100	1×10^2			
	Bacillus muralis	1	100	1×10^2			
	Citrobacter freundii	2	99.4	1×10^2			
	Comamonas terrae	n. d.	99.5	1×10^2			
	Dermacoccus sp.	n. d.	99.9	1×10^3			
	Escherichia sp. / *Shigella* sp.	2	99.2–99.9	1×10^3			
	Escherichia fergusonii	2	99.6–99.7	3×10^3			
	Klebsiella michiganensis	n. d.	99.5	1×10^2			
	Lysinibacillus macroides	n. d.	99.4	1×10^2			
	Macrococcus caseolyticus	2	100	1×10^2			
	Microbacterium paraoxydans	1	99.6–99.8	1×10^3			
	Staphylococcus warneri	1	98.9-100	3×10^2			
Clone Library	*Achromobacter anxifer/dolens*	n. d.			1	99.8	0.5
	Acinetobacter baumannii	2			3	99.1–99.8	1.6
	Clostridium sp.	n. d.			65	100	34.9
	Enterococcus faecium	2			2	99.2–99.5	1.1
	Enterococcus gallinarum	2			5	98.7–99.7	2.7
	Enterococcus lactis	n. d.			11	98.7–99.6	5.9
	Jeotgalicoccus coquinae	n. d.			1	99.7	0.5
	Macrococcus brunensis	1			1	99.5	0.5
	Romboutsia ilealis	n. d.			1	99.1	0.5
	Staphylococcus capitis/caprae	1/2			1	99.8	0.5
	Staph. delph./intermed./pseudintermed.	1/2/2			5	99.6-100	2.7
	Staphylococcus epidermidis	2			2	99.6-100	1.1
	Staphylococcus hominis	2			1	99.3	0.5
	uncultured				11	< 98	5.9
Fungi	*Aspergillus fumigatus*	2		1×10^3			
	Candida sp.	n. d.		2×10^3			
	Trichoderma koningii	n. d.		2×10^2			
	Trichoderma longibrachiatum	n. d.		2×10^2			
	Trichoderma sp.	n. d.		3×10^3			

Discussion

In this study workers' exposure to airborne microorganisms in a chicken and turkey hatchery was investigated. Despite rigorous hygiene measures including egg sanitization and incubator disinfection, high concentrations of airborne microorganisms were detected both by colony count and total cell count in both hatcheries during work activities with poult handling. In contrast, tasks without poult processing and outside air displayed far less levels of microbial air contamination indicating poults and eggshells as a major source of hatchery bioaerosols. Moreover, comparison of levels of airborne microorganisms detected during different tasks revealed no substantial differences. In contrast to occupational chemical and physical hazards no exposure limit has been defined yet for biological agents, mainly due to the problematic establishment of dose-effect relationships. However, Dutkiewicz et al. [30] and Gorny et al. [31] suggested an occupational exposure level (OEL) of 1 x 10^5 cfu m^{-3} for mesophilic bacteria at agricultural workplaces. The maximum concentration of 5.4 x 10^5 cfu m^{-3} detected in the investigated chicken hatchery exceeded the OEL but remained under in the turkey hatchery (1.4 x 10^4 cfu m^{-3}). These concentrations were lower than in a duck hatchery during sorting of ducklings (7 x 10^6 cfu m^{-3}) [7] but comparable to those determined in two chicken hatchery studies (1.3 x 10^5 cfu m^{-3}, 3.0 x 10^5 cfu m^{-3}) [32, 33] or in turkey (7 x 10^5 cfu m^{-3}) [7] and duck houses (3 x 10^5 cfu m^{-3}) [20]. In addition to detection of airborne bacteria, airborne moulds were detected by cultivation as well. Although no moulds were detected in the chicken hatchery in absence of poults, in both hatcheries concentrations during poult handling were comparable to those in outside air indicating that moulds present in workplace air did not originate solely from poults or eggshells. However, comparison of cultivation derived quantification from different studies and laboratories is questionable due to potentially different microbial communities as well as different sampling devices and culture media used for analysis. Therefore, further proceedings in standardization of characterization of microbial exposure are required. As observed in this study, results from colony count and total cell count can differ by several orders of magnitude. For this reason, approaches like cultivation independent DAPI staining of bioaerosol samples provide more reliable quantification [34] but only few studies report total cell counts from work places in poultry production. Martin et al. [16, 20] quantified microorganisms in duck house and duck hatchery air by total cell count and detected up to 8 x 10^7 cells m^{-3} and 2 x 10^7 cells m^{-3}, respectively, whereas levels in the here investigated chicken and turkey hatchery were substantially lower. Albeit both hatcheries processed comparable numbers of poults (24.000 – 32.000) at removal days, concentrations of airborne microorganisms in the chicken hatchery were higher. Next to

poultry specific differences, these findings maybe also reflect different efforts in hygiene management. In contrast to the chicken hatchery, access restrictions and attention to basic hygiene measures were more stringent in the investigated turkey hatchery.

Next to quantification also the identity of microorganisms is crucial for characterization of occupational microbial exposure. Analysis of the composition of bacterial communities in chicken and turkey hatchery air by generation of 16S rRNA gene clone libraries and identification of isolates revealed an extensive similarity on the genus level and in species richness. In both hatcheries *Enterococcus*, *Clostridium*, *Staphylococcus* and *Acinetobacter* were identified as predominant genera. Except for *Clostridium*, these genera were also detected in workplace air of two Polish chicken hatcheries [32, 33] and a German duck hatchery [16]. However, anaerobic cultivation or generation of 16S rRNA clone libraries was not reported by the Polish investigators. For this reason occurrence of strictly anaerobic *Clostridium* spp. could not be excluded in these hatcheries, especially since this genus accounts for major fractions of the microbiota of the gastrointestinal tract in domestic chickens [35, 36], turkeys [36, 37] and ducks [38, 39]. Furthermore, this genus illustrates a limitation of 16S rRNA gene analysis for the purpose of identification because due to high similarity within the genus *Clostridium* no species assignment could be performed. Analysis of bacterial diversity revealed similar numbers of identified genera in the chicken (18) and turkey hatchery (20). Compared to the diversity detected in turkey (28 genera) [7] and duck houses (29 genera) [20], hatcheries display a lower diversity maybe indicating less sources of bacterial contamination and more homogenous habitats. Furthermore, bacterial community compositions in hatcheries display significant differences compared to communities in poultry sheds. In contrast to hatcheries, predominant species in turkey and broiler sheds were assigned to the genera *Lactobacillus*, *Staphylococcus*, *Aerococcus*, *Corynebacterium* or *Brevibacterium* [7, 40, 41].

When establishing risk assessments regarding bacterial exposure an assignment on the genus level is not sufficient. According to EU directive no. 2000/54/EC several national guidelines for classification of microbial species in regard to levels of infection risk were established, e.g. German TRBA 466, Swiss BAFU list or Polish ordinance no. 81 sec. 716. In the investigated chicken and turkey hatcheries numerous identified species have been classified as risk group 2 microorganisms (TRBA 466) and are well-known bacterial and fungal pathogens (Tab. 2). In both hatcheries *Enterococcus* species and in particular *E. faecalis* accounted for large proportions in bacterial community. As *Clostridium* spp., also *Enterococcus* spp. are part of the natural poultry intestinal microbiome [35, 36, 42, 43] and were commonly

identified in poultry production [44-47]. Clinical significance of these species is defined by frequent occurrence as causative agents of nosocomial infections (Tab. 2). Further large fractions in bacterial communities of hatchery air were assigned to the genus *Staphylococcus* and as reported by several investigators hatchery workers are frequently exposed to high concentrations of airborne *Staphylococcus* spp. and in particular to *S. aureus* [32, 33, 48], one of the most important human pathogens and capable of causing a wide range of diseases (Tab. 2). Moreover, the risk of infection and spread into community is potentially increased by nasal carriage among employees in livestock industry which is associated to occupational exposure [49, 50]. While *S. aureus* dominated in the chicken hatchery, this species was not identified in the turkey hatchery where a major occurrence of non-pathogenic *S. lentus* was detected. Other potential pathogenic staphylococci species (Tab. 2) assigned to risk group 2 as *S. chromogenes* and *S. agnetis/hyicus* (chicken hatchery) as well as *S. epidermidis*, *S. hominis* and *S. capitis/caprae* (turkey hatchery) accounted for low fractions only. Furthermore, a cluster of 16S rRNA gene sequences (2.7%) within the generated turkey hatchery clone library was most closely related to *S. delphini*, *S. intermedius* and *S. pseudintermedius*. This clone library cluster was also detected by Martin et al. [16] in the aforementioned duck hatchery but accounted for 22%. These findings demonstrate that despite extensive similarity of bacterial communities on the genus level in different hatcheries major differences on the species level are detectable. Next to different species of the genera *Enterococcus*, *Clostridium* and *Staphylococcus*, *A. baumannii* was detected in both hatcheries. Relevance of *A. baumannii* as a pathogen is defined by frequent appearance as causative agent of nosocomial infections [51]. Regarding occupational health risks in livestock industry, a cause of concern is the emergence of *A. baumannii* in poultry production as isolates has been recovered from poultry feathers [52] and in hatchery air [16, 17, 33]. Moreover, Skórska et al. [18] reported significant more precipitin reactions to *A. baumannii* antigens in a group of chicken hatchery workers than in the reference group. However, they did not detect a correlation to the prevalence of work-related symptoms.

Furthermore, for an adequate risk assessment on the mentioned predominant bacteria in hatchery air, next to their pathogenicity also potential antibiotic resistance has to be considered. In this context the increasing occurrence of multi-drug resistant strains of *E. faecalis* [47, 53], *S. aureus* [54, 55] and *A. baumannii* [56] in poultry production may reduce therapy options in the case of occupational infections.

Besides the major bacterial genera in hatchery air, numerous different species were detected that accounted for very low fractions only. These comprise various gram negative species,

including *Enterobacteriacea,* as *Klebsiella* spp., *Enterobacter* spp., *Escherichia fergusonii*, *Citrobacter freundii*, *Chryseobacterium gleum*, *Leclercia adecarboxylata*, and *Stenotrophomonas maltophilia* which have been classified as risk group 2 microorganisms and often have relevance as causative agents of hospital-acquired infections especially for immunocompromised persons (Tab. 2). Moreover, for several identified species as *Massilia timonae*, *Kocuria rhizophila*, *Microbacterium paraoxydans*, *Nocardia globerula* or *Staphylococcus warneri* infectiousness has been reported despite classification as risk group 1 microorganisms (Tab. 2). Since several species were detected by the cultivation approach only, limitations of the molecular cloning approach become apparent. Construction of two clone libraries based on 330 and 186 sequences implicates that only sequences with a frequency of at least 0.3 and 0.5%, respectively, were detectable in theory.

In addition to bacteria, also mould isolates were recovered from hatchery air, albeit concentrations in hatchery air did not differ substantially from those in outside air. Abundant isolates in both hatcheries were identified as species of the genera *Aspergillus* and *Penicillium*. In particular *A. fumigatus* (risk group 2) is known as causative agent of human allergic respiratory disease (Tab. 2) and is frequently found in poultry environments [57-59].

Table 2 Identified bacterial and fungal pathogens in a chicken and a turkey hatchery.

Bacterial Pathogens	Disease	References
Acinetobacter baumannii	nosocomial infections, pneumonia, bacteremia, meningitis	[51, 60, 61]
Chryseobacterium gleum	respiratory tract infections, pyonephrosis	[62-64]
Citrobacter freundii	nosocomial infections, bacteremia, gastroenteritis	[65-68]
Enterobacter asburiae	soft tissue infections, bloodstream infections, pneumonia	[69-71]
Enterococcus faecalis/faecium	nosocomial infections, urinary tract infections, wound infections	[72-74]
Enterococcus gallinarum	bacteremia, endocarditis	[75-77]
Escherichia coli/fergusonii	gastroenteritis, urinary tract infections, meningitis	[78-82]
Klebsiella spp.	bloodstream infections, liver abscess, pneumonia	[83-86]
Kocuria rhizophila	catheter-related bacteremia	[87-90]
Leclercia adecarboxylata	bacteremia, wound infections	[91, 92]
Massilia timonae	bloodstream infections, wound infections	[93-96]
Microbacterium paraoxydans	catheter-related bacteremia, peritonitis	[97-100]
Nocardia globerula	bacteremia, keratitis	[101, 102]
Shigella dysenteriae	dysentery	[103, 104]
Staph. aureus	nosocomial infections, respiratory infections, wound infections	[105-107]
Staph. capitis/epidermidis/hominis	bloodstream infections, cardiovascular infections	[108-112]
Staph. warneri	bacteremia, osteomyelitis, endocarditis	[113-116]
Stenotrophomonas maltophilia	bloodstream infections, pneumonia	[117, 118]
Fungal Pathogens	Disease	
Aspergillus fumigatus	respiratory tract infections, allergic aspergillosis	[79, 119]
Aspergillus sydowii	onychomycosis	[120, 121]
Aspergillus versicolor	allergic respiratory disease, onychomycosis	[122-125]
Penicillium spp.	toxin formation, allergic respiratory disease, infections	[126-131]
Scopulariopsis brevicaulis	onychomycosis, skin infections, deep tissue infections	[132-135]
Trichoderma spp.	invasive infections	[136-138]

Conclusions

Employees in poultry hatcheries are exposed to high concentrations of airborne bacteria, in particular during tasks with contact to contaminated poults and eggshells. Comparison of levels of airborne bacteria in both hatcheries revealed higher concentrations in the chicken hatchery conceivably due to chicken specific characteristics as well as less attention to basic hygiene measures. Analysis of the bacterial community composition in the investigated chicken and turkey hatchery revealed an extensive similarity on the genus level indicating the presence of similar ecological conditions in both hatcheries independently from the poultry species. The identified predominant genera *Enterococcus*, *Clostridium*, *Staphylococcus* and *Acinetobacter* as well as various *Enterobacteriaceae* comprise well-known clinical pathogens but their actual contribution to pathogenesis of occupational respiratory disorders remains to be investigated. In consideration of these results further efforts are required to effectively reduce workers' exposure to potentially harmful bioaerosols. Next to technical measures that minimize levels of airborne bacteria, an effective personal breathing protection is essential. Furthermore, characterization of the exposure by different approaches revealed substantial differences between cultivation dependent quantification and total cell counts as well as between cultivation dependent identification and the molecular cloning approach. These findings emphasize the need for a further advancement in standardization of sampling and sample processing.

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Manuscript II

Eggshells as a Source for Occupational Exposure to Airborne Bacteria in Hatcheries

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3.2 Detection of Workers' Antibody Responses to Airborne Bacteria (III-V)

Manuscript III

Automated Image Analysis for Determination of Antibody Titers Against Occupational Bacterial Antigens

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Manuscript IV

Hatchery Workers‘ IgG Antibody Profiles to Airborne Bacteria

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Manuscript V

Use of Laser Microdissection for Identification of Immunogenic Bacteria in Bioaerosols

unpublished

Use of Laser Microdissection for Identification of Immunogenic Bacteria in Bioaerosols

Abstract

Long term inhalation of agricultural bioaerosols may cause a wide range of adverse health effects. However, etiology and in particular microorganisms' potential role in pathogenesis still need to be elucidated. Thus, identification of immunogenic bacteria in complex bioaerosol samples may lead to further comprehension. The novel approach presented here aimed at development of a protocol for 16S rRNA gene analysis of bacterial cells separated from complex bioaerosol samples by Laser Microdissection and Pressure Catapulting (LMPC). Although technical key issues like an appropriate base material or mode of cell lysis have been established successfully, PCR based amplification of 16S rRNA genes from microdissected cells could not be accomplished until now and remains to be further optimized.

Introduction

Occupational exposure to complex agricultural bioaerosols is related to an increased risk for respiratory disorders, particularly a decline in lung function, chronic obstructive pulmonary disease and hypersensitivity pneumonitis [1-6]. However, pathogenesis is not well understood and causative agents are not precisely defined. Therefore, analysis of specific host immune responses against microbial antigens may provide deeper insights. In occupational health research immunological methods like agar immunodiffusion tests [7] or enzyme immunoassays [8] have been applied to detect antibody responses after bioaerosol exposure. Nevertheless, application of these approaches is restricted to a selection of cultivable microbial species occurring at certain workplaces but their true impact on respiratory tract-bioaerosol interactions is often not clear and has to be derived from clinical empirical knowledge. In contrast to pure cultures, employment of complete bioaerosol samples as test antigens covers the entire proportional microbial exposure at specific workplaces including eukaryotic cells of fungi or protozoa. As reported by Brauner et al. (Manuscript IV) antibody binding to serum incubated airborne antigens can be easily visualized by indirect immunofluorescence and allows determination of workers' immunoreactivity to occupational bioaerosols although individual cell identity is unknown. Thus, identification of antibody labelled bacterial cells would conceivably provide profound knowledge about immunogenic species in agricultural environments and would facilitate specific investigations concerning bacterial involvement in work related respiratory disorders. In this context employment of LMPC for analysis of single cells is a promising approach towards further comprehension. Since cells are viewed and photographed during isolation, LMPC provides a high degree of certainty that only target cells are selected. The LMPC technology couples a pulsed UV-A laser with a standard microscope and allows ablation of material (laser microdissection) on a micron-sized spot. By using the same laser separated cells can be lifted up by a photon force (laser pressure catapulting) and captured in a collection device thereby enabling non-contact preparation of pure samples [9]. Developed at the National Cancer Institute of the National Institute of Health (NIH), Bethesda (USA), by Michael Emmert-Buck and colleagues [10], LMPC was originally employed for identification of genes specific for malignant cells [11]. However, since then this technique has been used for widespread applications, for example in plant physiology [12], forensics [13] or pathology [14]. Next to analysis of eukaryotic cells or tissues LMPC also enabled characterization of host-bacteria interactions [15, 16] or phylogenetic analysis of specific bacteria in aquatic samples [17]. As a common method to visualize specific bacterial cells in complex samples, fluorescence *in situ* hybridization

(FISH) is often combined with LMPC techniques [18, 19]. Nevertheless, to our knowledge, this is the first approach aiming at identification of serum incubated and immunofluorescence labelled bacterial cells in bioaerosol samples for comprehension of the immunogenic potential of airborne bacteria.

Material and Methods

Bacteria and Antibodies

For establishment of a LMPC protocol *Pseudomonas aeruginosa* ATCC 33358 (serotype O11) was used. Furthermore, specifity of monoclonal anti-*P. aeruginosa* O11 lipopolysaccharide antibody (IgM, KBPA 101; Kenta Biotech, Switzerland) [20, 21] and anti-*Staphylococcus aureus* lipoteichoic acid antibody (IgG, KBSA 302; Kenta Biotech, Switzerland) were tested on various bacterial species. Bacterial suspensions were generated by harvesting colonies from casein-soy agar (Carl Roth, Germany) into 0.9% NaCl. Subsequently, bacteria were inactivated and fixed by addition of ethanol to a final concentration of 50% (vol/vol).

Laser Microdissection and Pressure Catapulting

LMPC was performed with a Palm MicroBeam system in combination with a Palm RoboMover controlled by Palm RoboSoftware v2.0 (Palm Microlaser Technologies GmbH, Bernried, Germany). The MicroBeam system included an Axiovert 200 M microscope (Carl Zeiss Microimaging GmbH, Germany) equipped with a 100-W mercury lamp. Bacteria on base materials were inspected at x 400 (dry), x 630 (dry) or x 1000 (oil immersion) magnification. Sections were either catapulted in especially adapted collection devices filled with an adhesive material for buffer-free sampling (Adhesive Caps, Carl Zeiss Microimaging, Germany) or into lids of standard 0.2 ml PCR tubes (Kisker Biotech, Germany). Lids of PCR tubes were filled with 3 µl Tris EDTA (TE) buffer. Before use TE buffer was incubated with DNase I (0.07 U μl^{-1}) for 12 min at room temperature followed by an inactivation step (95°C for 15 min). The lifting process of laser sections was checked by visual inspection of the lids at x 100 magnification. Contamination-free LMPC was ensured by a glass box casing the MicroBeam system as well as by application of UV light and DNA removing reagents.

Base Materials for LMPC

For LMPC different base materials were tested. Polycarbonat filters (Ø 25 mm, black, 0.2 µm pore size): Cyclopore (Whatman, UK), Nuclepore (Whatman, UK), Isopore (Millipore, Germany). Microscopic slides (24 x 76 mm): 0.17 mm PET-membrane covered membrane slides (Carl Zeiss MicroImaging, Germany), PET-membrane covered frame slides (Carl Zeiss MicroImaging, Germany), 0.17 mm glass slides (Hecht-Assistent, Germany). Adherence of bacteria on polycarbonate filters was performed by using a filtration unit (Ø 20 mm, 30 ml; Sartorius, Germany). For adherence and fluorescence staining of bacteria on microscopic slides, a circle (Ø 1 cm) was drawn with a water-repellent barrier marker (Carl Roth, Germany) and suspensions containing bacteria were pipetted (20 µl) in the circle for air drying.

Fluorescence Staining of Bacteria

For establishment of the LMPC procedure *P. aeruginosa* was either stained solely with different concentrations (0.1–10 µg ml^{-1}) of 4',6-diamidino-2-phenylindole (DAPI) or additionally by indirect immunofluorescence (IIF). IIF was also performed for specifity testing of anti-bacterial antibodies. The IIF procedure was either conducted on polycarbonate filters or on microscopic slides. IIF on polycarbonate filters was performed by inserting filters into steel syringe filter holder (Sartorius, Germany) and all buffers were applied via syringes and removed using a vacuum manifold (J. T. Baker, USA). IIF assays were performed according to a modified protocol described originally by Jenzora et al. [22]. After incubation with blocking agent [5% goat serum (Sigma-Aldrich, USA) and 1% bovine serum albumin (Carl Roth, Germany) in PBS] for 30 min at room temperature, primary antibody (1 µg ml^{-1} KBPA 101 or KBSA 302) was added and incubated for 60 min at 37°C. After washing, Alexa Fluor 488 conjugated goat anti-human [IgM or IgG secondary antibody (1 µg ml^{-1}); Dianova, Germany] and DAPI were added for 30 min at 37°C followed by final washing. In order to avoid fluorescence fading, an anti-fading reagent (AF1, Citifluor, UK) was used for antibody specifity testing.

Cell lysis and DNA Extraction

In order to test the applicability of various commercial DNA preparation kits (Tab.1) for DNA extraction from minimal cell numbers, serial dilutions of *P. aeruginosa* suspensions were used. In addition, a modified cell lysis protocol originally described by Gloess et al. [17] was tested for cells suspensions and bacteria loaded laser sections. Cell suspensions (10 µl) were pipetted into the lids of 0.2 ml PCR tubes or 3 µl buffer were used for LMPC. Fluids in the lid were spun down for 5 min at 16.000 x g. Subsequently, tubes were sonicated for 2 min, followed by 5 cycles freeze-thaw (-196°C/98°C) and final centrifugation (2 min, 16.000 x g).

Table 1 Commercial kits tested for applicability in DNA extraction of minimal cell numbers.

Kit	Manufacturer	Application
GenElute Plant Genomic DNA Kit	Sigma-Aldrich, USA	plant tissue, (bacteria)
Bio 101 Kit	MP Biomedicals, USA	soil samples
QIAamp DNA FFPE Tissue Kit	Qiagen, Germany	formalin-fixed, paraffin-embedded tissue
QIAamp DNA Micro Kit	Qiagen, Germany	small amounts of blood or tissue
BlackPREP FFPE DNA Kit	Analytik Jena, Germany	formalin-fixed, paraffin-embedded tissue
InnuPREP Bacteria DNA Kit	Analytik Jena, Germany	gram positive and negative bacteria
InnuPREP DNA Micro Kit	Analytik Jena, Germany	small amounts of blood or tissue
InnuPREP Forensic Kit	Analytik Jena, Germany	forensic samples
PeqGOLD Bacterial DNA Kit	PeqLab, Germany	gram positive and negative bacteria
PrepMan Ultra Preparation Reagent	ThermoFisher, USA	fungi, bacteria, blood

16S rRNA gene amplification

In order to verify a successful cell lysis from minimal cell numbers, 16S rRNA genes were partially amplified. Amplification was performed with universal bacterial primers fD1 and rP1 primer [23] according to a protocol originally described by Gloess et al. [17]. The reaction mixture (15 µl) contained 9.2 µl water, 1.5 µl 10x *Taq* polymerase buffer, 0.9 µl $MgCl_2$ (25 mM), 1.5 µl dNTP mix (2 mM), 0.15 µl BSA (20 mg ml^{-1}), 0.3 µl of each Primer (10 pmol $µl^{-1}$) and 0.075 µl HotStar *Taq* polymerase (5 U $µl^{-1}$; Qiagen, Germany). In order to avoid contaminations with bacterial DNA, 0.085 µl DNase I (8.2 U $µl^{-1}$; AppliChem, Germany) were added to the reaction mixture and incubated for 12 min at room temperature followed by a DNase I inactivation [24] and polymerase activation step at 95°C for 15 min. To each reaction mixture 1 µl of cell lysate was added. The PCR was performed in a iQ5 Cycler (Biorad, Germany) in 48 amplification cycles with a program as follows: initial denaturation

at 95°C for 3 min, denaturation 95°C for 45 s, annealing at 57.3°C for 45 s, extension at 72°C for 1 min, final extension at 72°C for 15 min.

Multiple Displacement amplification (MDA)

In order to pre-amplify bacterial genomic DNA to generate sufficient amounts for PCR amplification, MDA was applied to cell lysates from serial dilutions as well as LMPC samples. For MDA the REPLI-g Mini Kit (Qiagen, Germany) was used according to the manufacturer's instructions. Amplified DNA from MDA was visualized after gel electrophoresis (1.0% agarose) by staining DNA with ethidium bromide (50 µg l^{-1}) and amplified DNA from PCR was analyzed and documented with a microchip electrophoresis system using the DNA-2500 Reagent Kit (MCE-202 MultiNA, Shimadzu, Japan).

Results and Discussion

Specifity Testing

This study aimed at establishment of a LMPC procedure for the identification of immunogenic bacteria in occupational bioaerosols. Significance of the results will depend crucially from a reliable separation of antibody bound bacterial cells from non-immunogenic cells. For this reason it was intended to validate the precision of cell separation by experimental spiking of bioaerosol samples with *P. aeruginosa* ATCC 33358 and *S. aureus* ATCC 25904. In order to label the target bacteria for LMPC indirect immunofluorescence with the specific monoclonal antibodies KBPA 101 (anti-*P. aeruginosa*, serotype O11) and KBSA 302 (anti-*S. aureus* lipoteichoic acid) was planned. Finally, separated cells were intended to be identified by 16S rRNA gene analysis. However, prior to spiking experiments adequate specifity of both antibodies for their target species had to be ensured to avoid false positive results during LMPC procedure. Otherwise it might be difficult to distinguish bacterial contamination from erroneously labelled and separated bacteria and validity of experimental spiking would be diminished. For antibody specifity testing an approach was chosen that has been already applied successfully in bacterial primer testing [25-27]. Antibody binding was tested (Tab. 2) on other strains of the same species, on other species of the same genus as well as on species of other genera. In order to visualize antibody binding, specifity testing was performed by using indirect immunofluorescence. For evaluation of the specifity it was assessed if a visible fluorescence was present or not. Antibody KBSA 302 was tested on two *S. aureus* strains as well as on eight *Staphylococcus* species and six gram positive species of other genera but no binding to other species was detected. Likewise,

antibody KBPA 101 was tested on different *P. aeruginosa* serotypes as well as on eight other *Pseudomonas* species and on four gram negative species of other genera. No binding to serotypes O1 (*P. aeruginosa* ATCC 33348) and O6 (ATCC 33354, DSM 1117) as well as to other *Pseudomonas* species was observed. Moreover, except for *Sphingomonas terrae* also no binding to species of other genera was detected. Binding of KBPA 101 to *S. terrae* may indicate a cross-reaction due to high antigenic similarity of lipopolysaccharides, a phenomenon often observed among gram negative bacteria [28, 29]. In addition to primary antibodies also the conjugated secondary antibody was tested on all strains to exclude unspecific binding. In consideration of these results, specifity of the two tested antibodies was assumed to be sufficient for application in spiking experiments with bioaerosol samples.

Table 2 Results from specifity testing of monoclonal antibodies KBSA 302 and KBPA 101 on various gram positive and gram negative species by indirect immunofluorescence. (+ binding, - no binding)

Gram positive species/strains	KBSA 302	Gram negative species/strains	KBPA 101
Staphylococcus aureus ATCC 25904	+	*Pseudomonas aeruginosa* O1, ATCC 33348	-
Staphylococcus aureus DSM 1104	+	*Pseudomonas aeruginosa* O6, ATCC 33354	-
Staphylococcus simiae DSM 17636	-	*Pseudomonas aeruginosa* O11, ATCC 33358	+
Staphylococcus caprae DSM 20608	-	*Pseudomonas aeruginosa* O6, DSM 1117	-
Staphylococcus capitis DSM 20326	-	*Pseudomonas otitidis* DSM 17224	-
Staphylococcus warneri DSM 20316	-	*Pseudomonas resinovorans* DSM 21078	-
Staphylococcus hominis DSM 20328	-	*Pseudomonas alcaligenes* DSM 50342	-
Staphylococcus cohnii DSM 20260	-	*Pseudomonas peli* DSM 17833	-
Staphylococcus saprophyticus DSM 20229	-	*Pseudomonas mendocina* DSM 50017	-
Staphylococcus schleiferi DSM 4807	-	*Pseudomonas nitroreducens* DSM 14399	-
Microbacterium aerolatum DSM 14217	-	*Pseudomonas stutzeri* DSM 5190	-
Bacillus thuringiensis DSM 2046	-	*Pseudomonas segetis* DSM 18913	-
Jeotgalicoccus aerolatus DSM 22420	-	*Escherichia coli* DSM 1103	-
Arthrobacter roseus DSM 14508	-	*Chryseobacterium gleum* DSM 16776	-
Corynebacterium kroppenstedtii DSM 44385	-	*Stenotrophomonas maltophilia* DSM 50170	-
Enterococcus faecalis DSM 2570	-	*Sphingomonas terrae* DSM 8831	+

Establishment of the LMPC Procedure

For a precise separation of antibody labelled bacteria from complex bioaerosol samples, base materials have to satisfy different demands in regard to optical features as well as to LMPC practicability. That means base materials should enable microscopy and laser dissection at high magnification (x 1000) and should provide high contrast for fluorescence stained (DAPI and AF 488) but uncoverslipped specimens for a reliable discrimination between labelled and non-labelled bacteria. In order to identify appropriate materials that combine the required properties several different base materials were tested (Tab. 3). Black polycarbonate filters are employed for counting DAPI stained microorganisms in bioaerosol samples from agricultural workplaces [30, 31]. Moreover, Gloess et al. used polycarbonate filters for LMPC of polyphosphate-accumulating bacteria in environmental samples [17]. Adherence of bacteria on filters is easily performed by filtration and is advantageous due to the even distribution of cells on the filter surface. For laser cutting the filters had to be supported by a glass slide located beneath the filter (Fig. 1A). However, in this arrangement the filter surface was not completely in contact with the glass support. In consequence the free working distance of the 100x objective was exceeded and microscopy could be performed at x 630 or x 400 magnification only. Furthermore, two (Cyclopore, Nuclepore) of the three different polycarbonate filter types used in this study exhibited strong background fluorescence and bacteria on these filters displayed a low fluorescence intensity (Fig. 2). A better optical performance was provided by the Isopore filter but this material could not be cut completely by the laser, most likely due to its higher thickness. An additional approach was adapted from plant physiology research as membrane free glass slides were utilized for microdissection and catapulting of cells in plant tissues [12, 32]. Compared with polycarbonate filters, the used 0.17 mm glass slides provided high contrast of fluorescence stained bacteria (Fig. 3) but different from membrane slides the specimen is not stabilized (Fig. 1D). In consequence, laser shots were aimed directly on bacterial cells for lifting. On the one hand this procedure enabled the precise catapulting of single labelled cells but on the other hand targets might have been damaged or destroyed and a successful transfer into the lid above the slide could not be verified. Better results were obtained from membrane slides (Fig. 1B) and frame slides (Fig. 1C) as these materials provided sufficient optical properties (Fig. 2) and a stabilizing "backbone" for the specimen, so that sections could be easily detected in the lid after lifting (Fig. 3). Owing to these properties membrane slides are commonly used for a wide range of LCMP applications, e. g. selection of chloroplasts in plant material [33] or for analysis of frozen or formalin-fixed tissue sections [14, 15]. Since frame slides have to be supported by

an additional 0.17 mm slide when using oil immersion objectives, membrane slides are more convenient in handling and in consequence of base material testing, further analyses were performed with membrane slides only.

Next to base materials different devices for sample capture were tested. First, especially adapted 0.2 ml PCR tubes ("Adhesive Caps") were used. The lid of such a tube is filled with an adhesive gel to that loaded laser sections are adhered. This buffer-free approach avoids possible evaporation or crystal formation during prolonged sample capture. However, according to the manufacturer (Carl Zeiss MicroImaging) cell lysis is performed in an upside-down position but this position is prone to contamination and a transfer of the loaded membrane sections into the bottom of the vessel by centrifugation was prevented by strong adherence to the gel. Therefore, further LMPC analyses were performed by using standard PCR tubes with buffer filled caps.

Table 3 Comparison of different base materials for applicability in fluorescence microscopy and LMPC.

Material	Magnification	Fluorescence intensity	Background fluorescence	LMPC practicable?
Polycarbonate filter (Cyclopore)	x 630	weak	strong	yes
Polycarbonate filter (Nuclepore)	x 630	weak	strong	yes
Polycarbonate filter (Isopore)	x 630	medium	medium	no
Membrane slide	x 1000	strong	medium	yes
Frame slide	x 1000	strong	medium	yes
0.17 mm glass slide	x 1000	strong	weak	no

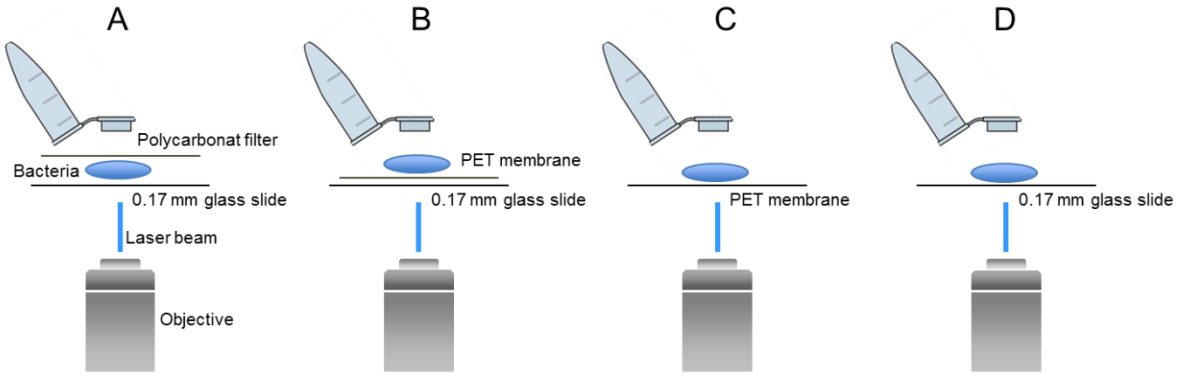

Figure 1 Scheme of sample arrangement for LMPC on different base materials: (A) polycarbonate filter, (B) membrane slide, (C) frame slide, (D) glass slide.

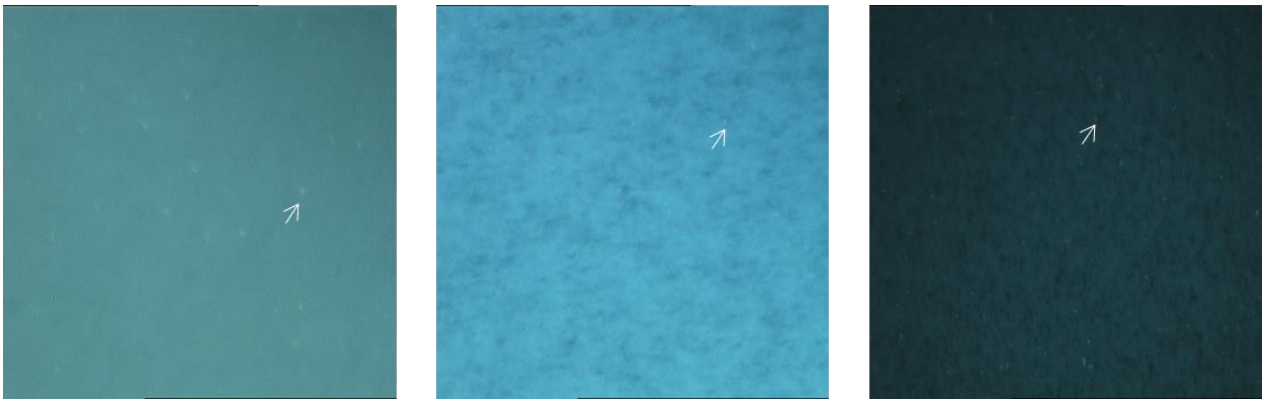

Figure 2 Microscopic images (x 630 magnification) of DAPI-stained *P. aeruginosa* cells on three different polycarbonate filter types. (arrows indicate bacterial cells; left, Cyclopore; middle, Nuclepore; right, Isopore)

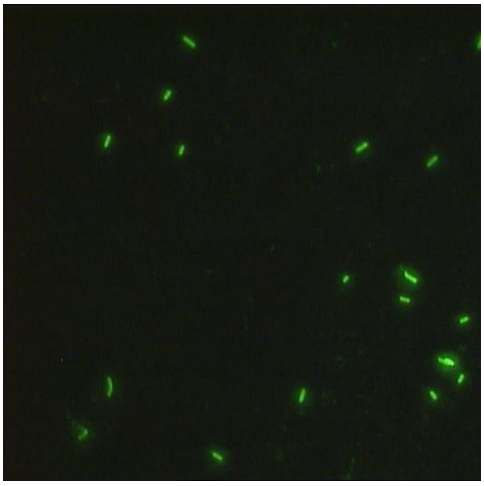

Figure 3 Microscopic images (x 1000 magnification) of AF488-stained *P. aeruginosa* cells on a 0.17 mm glass slide.

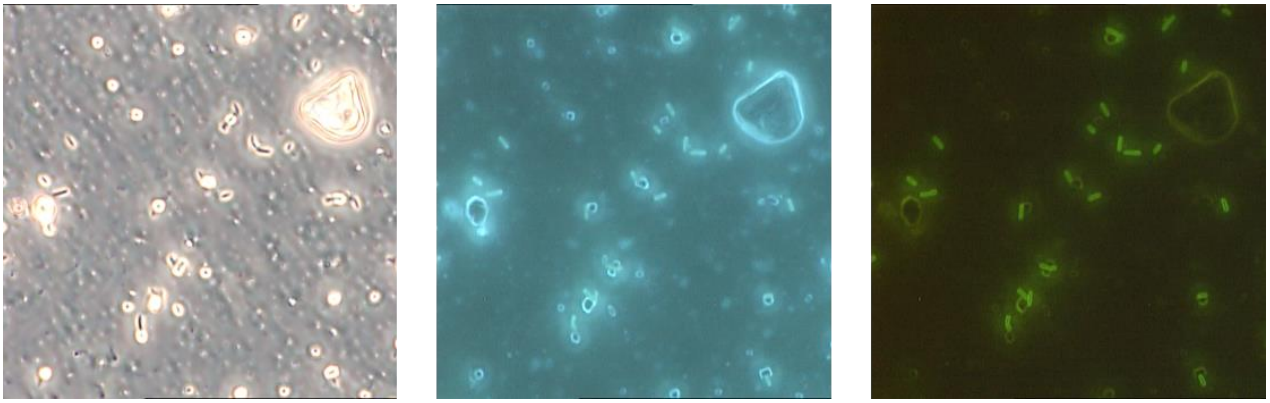

Figure 4 Microscopic images (x 1000 magnification) of *P. aeruginosa* cells on a membrane slide. (left, phase-contrast; middle, DAPI excitation; right, Alexa Fluor 488 excitation)

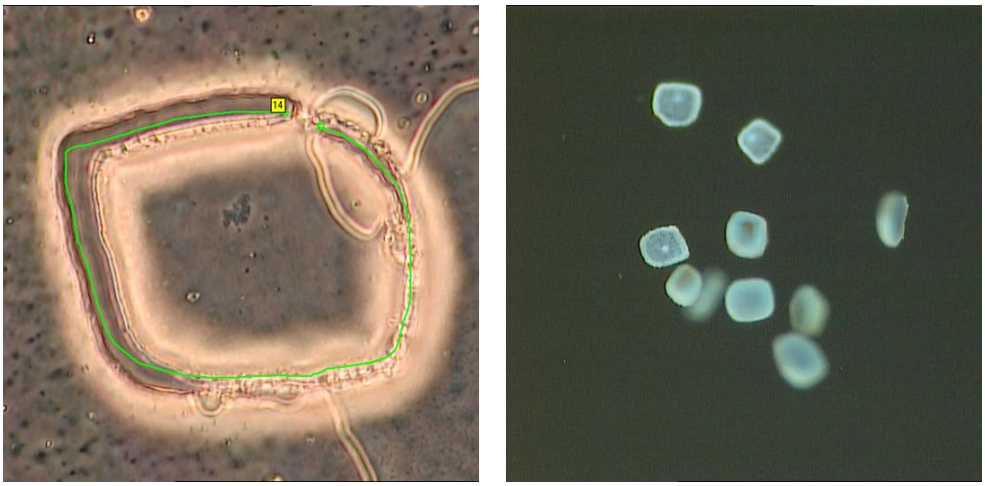

Figure 5 Microscopic images of the LMPC procedure. (left, x 1000 magnification, phase-contrast, laser cutting of a membrane slide; right x 100 magnification, DAPI excitation, laser sections after the lifting process in the buffer filled lid of an standard PCR tube)

Cell Lysis and 16S rRNA Gene Amplification

16S rRNA gene based identification of laser captured immunogenic bacteria in bioaerosol samples requires a specifically adapted protocol for cell lysis and PCR. In this context, determination of proportions of antibody-bound cells in serum incubated bioaerosol samples (Brauner et al. 2016, manuscript IV) revealed that at high serum dilutions only low numbers of bacteria display specific antibody binding. Moreover, only by low cell numbers in bioaerosol samples microdissection of single target cells is ensured and contamination by nearby non-labelled cells is avoided. For these reasons only low numbers (approx. 10–1.000) of bacterial cells are expected being available for molecular biological identification after LMPC procedure. First, various commercial kits were tested for DNA extraction from these minimal cell numbers in serial dilutions of *P. aeruginosa* (Tab. 1). Several of these kits have been specifically developed for DNA preparation from minimal amounts of eukaryotic cell material like forensic samples or paraffin embedded tissues whereas other kits focus on bacterial DNA extraction. When using these kits cell lysis was either performed by mechanical rupture with beads or by proteinase K and/or lysozyme digest. Common to all these kits are multiple liquid transfers which are prone to bacterial contamination [17] and may lead to loss of genomic DNA. Results obtained with commercial kits were dissatisfying since no PCR products could be reproducibly generated from minimal cell numbers (data not shown). In consequence a protocol originally described by Gloess et al. [17] was modified and tested. When using this protocol cell lysis was performed by sonication as well as repeated freeze-thaw cycles without any liquid transfers and DNA purification steps. Subsequently, the crude lysate was employed for 16S rRNA gene amplification. In order to

determine the lowest number of DAPI-stained and unstained cells which will enable generation of a detectable 16S rRNA gene PCR product after employment of this protocol, serial dilutions of *P. aeruginosa* cells were lysed. In absence of DAPI 1 to 10 cells were sufficient for a detectable amplification (Fig. 6). However, with increasing DAPI concentrations inhibition of the amplification reaction was intensified. In presence of 10 µg ml^{-1} DAPI, the standard concentration for determination of total cell counts in bioaerosols (VDI 4253, Bioaerosols and biological agents - Determination of total cell count by fluorescence analysis after staining with DAPI), at least 1.000 cells were required for a detectable amplification. Moreover, also a DAPI concentration of 5 µg ml^{-1} used by Gloess et al. [17] for bacteria staining required at least 1000 cells for a detectable amplification. In conclusion, the theoretical applicability of the protocol for lysis of low numbers of target cells after LMPC was confirmed but only DAPI concentrations of 1 µg ml^{-1} and below ensured detection of PCR products from at least 100 cells. Lower DAPI concentrations allowed a further reduction of the cell number but also lead to insufficient DAPI fluorescence.

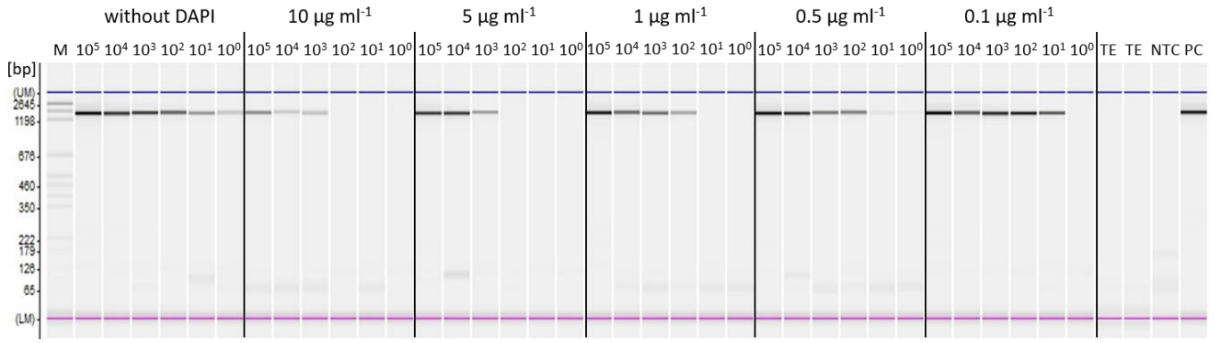

Figure 6 Dependence of PCR product concentrations from decreasing cells numbers in serial dilutions (10^0–10^5) of *P. aeruginosa* as well as from incubation of bacterial cells with decreasing DAPI concentrations (0.1–10 µg ml^{-1}). The virtual gel image displays PCR products from 16S rRNA gene amplification. One representative experiment out of two independent experiments is shown. (detection limit of MultiNA gel electrophoresis system: 0.2 ng $µl^{-1}$; M, marker; UM, upper marker; LM, lower marker; TE, Tris-EDTA lysis buffer without bacteria; NTC, no template control; PC, positive control)

After confirmation of the theoretical applicability of the cell lysis protocol for low cell numbers, *P. aeruginosa* loaded laser sections were employed for testing. Despite high cell numbers (1.000-8.000) PCR products were generated from a few samples only, indicating no reproducible amplification and no dependence from cell numbers (Fig. 7). In order to exclude potential inhibitory effects of PET membrane sections on cell lysis or PCR, 500 to 50.000 unstained cells were added to cell-free laser sections for lysis. In contrast to bacteria loaded laser sections, amplification products could be generated from these samples (data not shown). Most applications of LMPC techniques that target analysis of bacteria, focus on genomic identification of bacterial cells located in paraffin tissue sections that are mounted on membrane slides [15, 18, 34]. Therefore, it might be conceivable that adherence of bacteria on membrane slides by air drying has an adverse impact on cell lysis. However, it might be also conceivable that incomplete lysis or impaired amplification of 16S rRNA genes from adhered bacteria led to non-detection of low amounts of PCR product by gel electrophoresis.

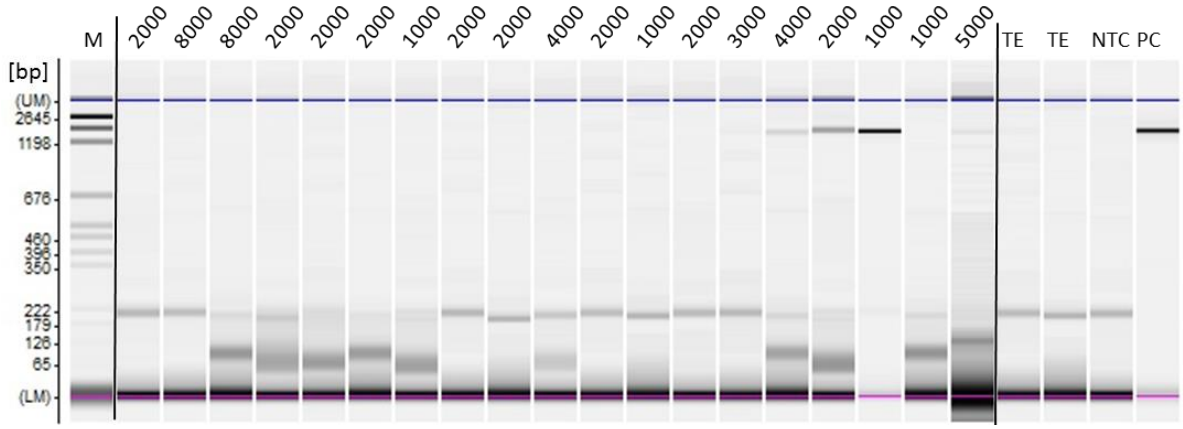

Figure 7 Virtual gel image of PCR products from 16S rRNA gene amplification of bacteria loaded laser sections (*P. aeruginosa*, varying cell numbers on 1-5 sections per sample). One representative experiment out of three independent experiments is shown. (M, marker; UM, upper marker; LM, lower marker; TE, Tris-EDTA lysis buffer without bacteria; NTC, no template control; PC, positive control)

In order to address these potential challenges, bacterial lysates from serial dilutions and loaded laser sections were employed for pre-amplification of genomic DNA by Multiple Displacement Amplification (MDA) [35, 36]. MDA uses random primers and the DNA polymerase from bacteriophage phi29 for a 30°C isothermal reaction. While DNA template is repeatedly copied by a branching mechanism previously made copies are concurrently displaced from the template strand. By applying this procedure, femtograms of DNA from a single bacterium can be amplified in micrograms of genomic DNA for further analyses [37, 38]. In order to generate sufficient genomic template for a detectable 16S rRNA gene PCR reaction, bacterial lysates from serial dilutions as well as from loaded laser sections were employed for pre-amplification. While MDA with lysates from serial dilutions (10-100.000 cells) generated detectable MDA products independently from cell numbers, amplification from loaded laser section (300–500 cells) was observed in three of eight samples only, indicating no reproducible amplification (Fig. 8 top). In order to assess whether MDA based pre-amplification generates appropriate template DNA for 16S rRNA gene amplification, MDA products from serial dilutions as well as from loaded laser section were applied for PCR. However, 16S rRNA gene amplification was detected only in samples containing MDA template from serial dilutions which may also additionally contained original DNA template from lysates (Fig. 8 bottom). Moreover, also no PCR products were generated from the three laser section samples that displayed MDA indicating that no 16S rRNA genes were pre-amplified. This might be a consequence of amplification bias, a common phenomenon in MDA reactions [37]. Nevertheless, as results from conventional PCR analysis also results

from MDA analysis indicated that adherence of bacterial cells to membrane laser sections impaired cell lysis and disabled 16S rRNA gene amplification.

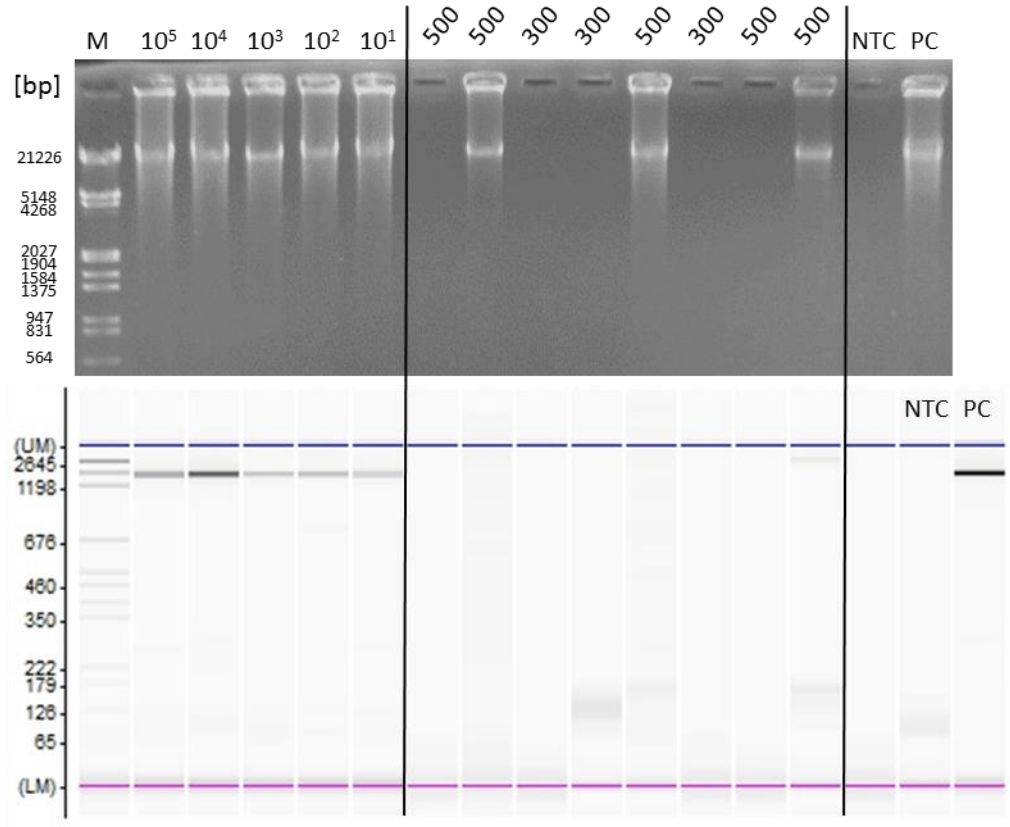

Figure 8 Top: Gel image of ethidium bromide stained DNA products from Multiple Displacement Amplification from varying cells numbers of *P. aeruginosa* in serial dilutions (10^1–10^5) and bacteria loaded laser sections (300–500). Bottom: Virtual gel image of corresponding PCR products from 16S rRNA gene amplification of MDA products. One representative experiment out of two independent experiments is shown. (M, marker; UM, upper marker; LM, lower marker; NTC, no template control; PC, positive control)

Conclusions

This study aimed at development of a protocol for laser microdissection and pressure catapulting as well as subsequent identification of immunogenic bacteria from agricultural bioaerosol samples. By establishing solutions for technical key issues, crucial requirements for validity testing of this new approach have been accomplished. In order to allow future assessment of the performance of this new approach in spiking experiments, adequate specifity of two monoclonal antibodies for their target species has been confirmed. Furthermore, testing of different base materials revealed that membrane slides combine appropriate features for microscopy and LMPC. Next to the LMPC procedure also different strategies for cell lysis and 16S rRNA gene analysis have been tested. Since commercial DNA extraction kits are prone to contamination and loss of genomic DNA, best results were obtained from a simple protocol using sonication as well as repeated freeze-thaw cycles. As confirmed with serial dilutions of bacterial suspensions this cell lysis protocol enables PCR based amplification of 16S rRNA genes from minimal cell numbers. However, for unknown reasons cell lysis from bacteria loaded laser sections did not allow a reproducible amplification. Since cell adherence to membrane sections is supposed to impair lysis, future efforts will address this issue.

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