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Evolution of a fungal ecosystem in a water distribution system to a positive bacterial biofilm subsequent to a treatment using essential oils

Évolution d'un biofilm à champignons filamenteux vers un biofilm bactérien positif après un traitement par huiles essentielles

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ABSTRACT

The present study aims to demonstrate the direct link between the microbial ecosystem of drinking water distribution systems and animal health in pig breeding. Based on a survey over 18 months, a treatment using essential oils proved to be efficient in increasing piglet health and zootechnical performance. Water pipe biofilms were monitored by laser scanning confocal microscopy, while zootechnical performance and health cost data were collected from professional organisations. In two representative monitored herds, it was observed that the drinking water distribution pipes, initially fouled by fungi, were replaced by a bacterial film while both veterinary costs and the total feed conversion ratio decreased. Essential oils may thus provide an efficient and sustainable alternative to the massive use of antibiotics for transforming an initial detrimental ecosystem to a positive biofilm.

Mots-clés:
Biofilms
Traitement de l'eau
Huiles essentielles
Imagerie par fluorescence

RÉSUMÉ

Cette étude vise à démontrer le lien direct qui relie l'écosystème microbien des canalisations d'eau potable d'un élevage de porc à la santé des animaux et à leurs performances zootechniques. Une étude de 18 mois menée sur deux fermes modèles montre l'effet bénéfique d'un traitement des canalisations par un mélange d'huiles essentielles. L'analyse des biofilms par microscopie laser confocale a révélé que la colonisation initiale des tuyaux par des champignons filamenteux était remplacée par un biofilm bactérien positif. Les huiles essentielles constituent donc une alternative durable à la prescription massive d'antibiotiques pour promouvoir un biofilm positif dans les réseaux d'alimentation d'eau de boisson des animaux.

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1. Introduction

Biofilms are usually described as microbial communities embedded in an exopolymeric matrix attached to biotic or abiotic surfaces. Such bacterial consortia are able to colonise virtually any environment, from natural biotopes to manmade instruments. Particular attention was paid to the biofilm colonization of water distribution systems [1], and particularly to the "last meters before the tap" which is often considered at risk for human and animal health [2]. Many problems in drinking water distribution systems (DSs) are microbial in nature, including biofilm growth [3], microbial mediated corrosion [4] and the persistence of pathogens [5]. While documented epidemiological studies that directly link disease occurrence with the level of DS pathogens are scarce, waterborne pathogens that are able to persist and reproduce in DS may cause infections of the gastrointestinal tract and the skin and lymph nodes of humans and animals.

The growth and persistence of pathogens is a major concern in DSs but the conventional approach for biological control, like maintaining a permanent disinfectant residual, is often ineffective in controlling microbial growth [6]. Loera-Muro and collaborators [7] have highlighted that viable *Actinobacillus pleuropneumoniae* is present in the drinking water of pig farms and occurs as biofilm communities as a strategy to survive in such an environment. Many treatments, chemical, physical or mechanical, are widely performed to eliminate these bacterial biofilms, but most of them are not very efficient [8–10]. Most zootechnical pathologies occurring in breeding units like pig farms cannot be prevented and/or cured by using regular antibiotics or other treatments. Besides, European microbial safety recommendations require stakeholders to reduce drastically the use of antibiotics which when spread in the environment cause major multi-resistance (Commission to the European Parliament and the Council, 2011). Several studies have highlighted that essential oils may have an important antimicrobial activity, especially against biofilms [11–13]. This alternative is therefore considered as a new approach to control microbial DS ecosystems. In this study, the microscopic evolution of biofilms in drinking water DSs of several pig farms was monitored during treatment using essential oils. This work used an unconventional approach because biofilm evolution was monitored in situ and in real time for fourteen months during which samples were taken from the drinking water distribution systems of pig farms. It therefore provides clues to real farm conditions which are often far from conditions reproduced in a laboratory. This study focuses on the impact of two commercial preparations (Dinamycol™ and Pigfast™) which are produced by the Dinastim Company. These formulations derive from long years on-field experiments which have demonstrated their benefit for pork zootechnical parameters. It has been also concluded that Pigfast must be applied after the Dinamycol treatment for an optimized effect. The time sequences (Fig. 2) and concentrations applied were observed to significantly improve the zootechnical results in farms where they were applied. Dinamycol™ prevents diarrhoea and improves the body's natural defences while Pigfast™ improves the daily live-mass gain.

2. Materials and methods

2.1. Experimental procedure

To monitor field biofilms accurately in farm DSs, samples were removed directly from pipes of the breeding room that convey the drinking water to the pigs from the drill water point to the trough (#7 in Fig. 1). Pipe samples were stored for a few hours in the farm's drinking water before being processed in the laboratory; this avoids varying conditions from altering the biofilm and allows for the characterisation of the undisturbed biofilms. In the following discussion, the pipe samples are called «coupons». In each farm, High Density PolyEthylene (HDPE) coupons are collected before, during and after the treatment with a cocktail of essential oils and hydro-alcoholic plant extracts in order to provide a dynamic view of the biofilm evolution over a three-month period. A new coupon replaced each pipe sample.

The samples were taken from different pig farms where the commercial Dinamycol™ and Pigfast™ formulations were added to the drinking water to cure recurrent zootechnical failures. Whereas Dinamycol only contains essential oils, Pigfast also contains oligo elements and hydro-alcoholic plant extracts. These two formulations have been demonstrated to exhibit complementary roles on the observed benefits. As evoked before, the protocols for water treatment in each breeding unit were designed by DINASTIM on an empiric basis to reduce or eliminate recurrent diarrhoea while improving the daily live-mass gain. The protocol here used has demonstrated the best results over years. The treatments were added to the animals' drinking water. Fig. 1 shows the farm's DS and the position of experimental coupons used for monitoring the biofilm. A dosing pump (#4 in Fig. 1) supplied either Dinamycol™ or Pigfast™ on demand at concentrations of 150 mL/m^3 and 300 mL/m^3, respectively. The DS monitoring covered nine farms over a period of fourteen months with sampling intervals similar for each farm. The following describes the biofilm evolution on two distinct farms which are representative of that observed in other breeding farms.

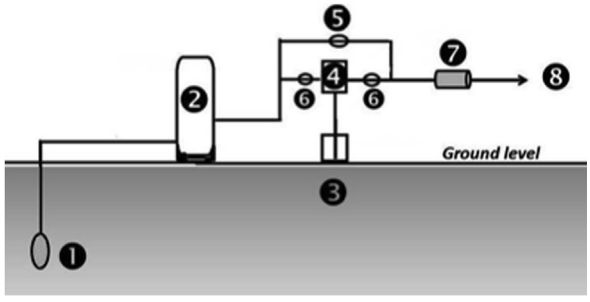

Fig. 1. Schematic view of the farm's water distribution system. 1: Borehole pump, 2: water buffering tank, 3: Dinastim™ or Pigfast™ delivery tank, 4: dosing distribution pump, 5: bypass, 6: pumps, 7: position of experimental coupons, and 8: to the troughs.

Fig. 2. Time sequence for the treatment of drinking water distribution systems. Units are weeks. Dinamycol™ (vertical hatching) is applied for eight weeks (W1 through W8), Pigfast™ (horizontal hatching) is applied for two days at the beginning of week ten (W10). Biofilm observations were made at T0, T8 and T13.

2.2. Epifluorescence and laser scanning confocal microscopy analyses

In order to analyse the biofilm formed on the inner surface of pipes and to evaluate its structural organization, laser scanning confocal microscopy (LSCM) observations were made using a Confocor 2 microscope (Zeiss, Germany). It comprises an Axiovert 200M microscope, an Ar laser as the light source and an LSM510 laser scanning unit. For fungal and bacterial colonisation and viability assessments, HDPE samples were cut into small squares of about 1 cm^2 on which was deposited 1 μL of a 1/1 mixture of two fluorophores: Syto9 (458 nm/475 nm) and propidium iodide (514 nm/570 nm) which make the Dead/Live kit (Molecular Probes Inc., USA). Additional observations were made by epifluorescence microscopy (BX60, Olympus) after DAPI (4′,6′-diamidino-2-phénylindole) labelling which is a DNA intercalate that allows for quantifying cell surface densities. It must be pointed that biofilm development is rather homogeneous in the tested pipes, this being likely due to the dynamic conditions which prevail in a water duct. Hence cell density evaluation must reflect the overall population.

2.3. Statistical analysis

A cell count was carried out using CELL D software (Olympus). Data for the three stages (T0, T8 and T13) are average values derived from the observations and counts of at least two images ($n \geq 2$) for each particular sample. Fungi were counted in terms of number of hyphae observed per surface unit.

3. Results

Table 1 and Figs. 3 and 4 all confirm that the initial biofilms are essentially composed of fungi where hyphae are prominent as shown by confocal laser and epifluorescence microscopy observations (Figs. 3A and 4A). However, it can be seen that, in breeding unit B, a significant dispersed bacterial population is also present while some bacterial clusters appear in close interaction with fungal hyphae, likely embedded in a polysaccharidic extracellular matrix. After 8 weeks of treatment with Dinamycol™, for both breeding units, the internal surfaces of pipes showed a more or less decreased but dying fungal population while a new bacterial population had set up (cf. Figs. 3B and 4B, Table 1). Hence, Dinamycol seems to have eliminated the initial development of fungi in the two breeding units studied. Concerning bacterial populations, this treatment induced distinct results for the two farms. In breeding unit A, there was a large bacterial onset from 0 (at T_0) to $14.7 \times 10^3 \pm 6.5 \times 10^3$ bacteria/mm^2 (at T_8) whereas in breeding unit B, the initial bacterial population decreased *10-fold* (see Table 1). A third observation was made at T_{13}, i.e., two weeks after the Pigfast treatment. At this stage,

Table 1
Time evolution of coupon biofilms subsequent to essential oil treatment. Representative observations for two breeding units are displayed. Stages are those shown in Fig. 2. Dead (red) and living (green) microorganisms are identified from their respective Dead/Live kit fluorescence. Cell densities are conventionally calculated by under-sampling microphotograph areas of equal surfaces and cells are counted by using the CELL D software. Three microphotographs are evaluated.

Stage	Population density (cells-hyphae/mm^2)	Viability (%) (fungi and/or bacteria)	Biofilm organization
Breeding A			
T0	$2.2 \times 10^3 \pm 0.9 \times 10^3$ fungi/mm^2	0% Fungi and Bacteria	The biofilm on the inner surface of the water pipe is mostly composed of dying fungi. (cf. Fig. 3A)
T8	$9.8 \times 10^3 \pm 2.3 \times 10^3$ fungi/mm^2 $14.7 \times 10^3 \pm 6.5 \times 10^3$ bacteria/mm^2	75% ± 8% Bacteria	The biofilm is mostly composed of living bacteria, a few dead hyphae are present. (cf. Fig. 3B)
T13	$17.5 \times 10^3 \pm 4.5 \times 10^3$ bacteria/mm^2	37% ± 12% Bacteria	Bacterial cell density is high. The biofilm is composed of some clusters of live bacteria while a significant number of cells are dead. No hyphae are observed. (cf. Fig. 3C)
Breeding B			
T0	$2.3 \times 10^3 \pm 1.4 \times 10^3$ fungi/mm^2 $58.5 \times 10^3 \pm 22.2 \times 10^3$ bacteria/mm^2	0% Bacteria 6% ± 5% Fungi	The biofilm is composed of both bacterial cells and hyphae. Small clusters are observed around hyphae which could be bacteria embedded in an extracellular matrix. (cf. Fig. 4A)
T8	$8.5 \times 10^3 \pm 2.4 \times 10^3$ fungi/mm^2 $6.25 \times 10^3 \pm 2.8 \times 10^3$ bacteria/mm^2	71% ± 47% Bacteria 13% ± 9% Fungi	Hyphae are in a larger amount and bacterial cell population is decreased by one log (cf. Fig. 4B)
T13	$29.9 \times 10^3 \pm 7.2 \times 10^3$ bacteria/mm^2	100% ± 0% Bacteria	Hyphae are no longer visible. The biofilm is composed of a high surface density of live bacterial cells. (cf. Fig. 4C)

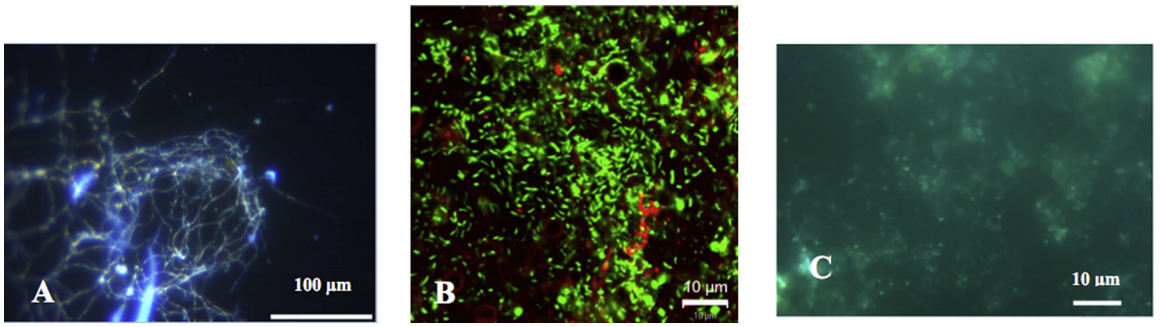

Fig. 3. Observations in confocal microscopy of the water pipe's inner surface (Breeding Unit A). A, T_0, B, T_8, C, and T_{13}. A and C are labelled with DAPI, B is labelled with the Dead/Live kit so that living cells exhibit green fluorescence whereas dead ones appear red.

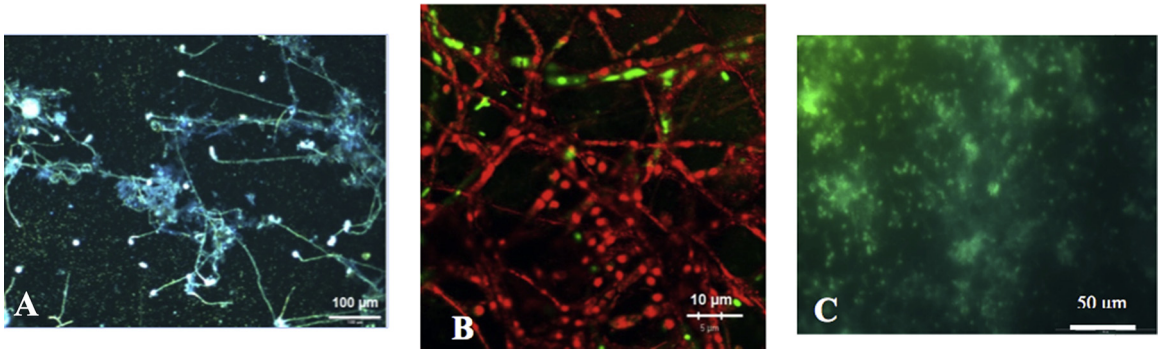

Fig. 4. Observations in confocal microscopy of the water pipe's inner surface (Breeding Unit B). Dying, symbols and legends are as in Fig. 3.

similar results were observed for both farms since fungal populations were eradicated from the inner surface of the DS pipes. At the same time, significant (≈ 100 mm^{-2}) cell surface densities were observed with various viabilities. This period corresponded to an observed increase in the zootechnical parameters since the livestock received lower amounts of antibiotics and animal losses were reduced. To sum up, fungi initially colonized farm DSs with associated bacterial populations to a greater or lesser degree in the investigated breeding units. Subsequent to the Dinamycol treatment, a decay of the fungal population was observed and, if existing, the initial bacterial populations also decreased. A second phase arose subsequent to the complementary Pigfast treatment which efficiently eradicated the fungal population to the benefit of possibly new bacterial populations. This phenomenon occurred within a few days (data not shown). Some fluorescence was observed suggesting that possibly new bacterial populations grow on a dead cellular substratum. Hence, this association of treatment actually alters the microbial population with associated positive effects for the livestock.

The evaluation of positive effects associated with the Dinamycol/Pigfast treatment was achieved by collecting long-term zootechnical and economic indices from local professional organisations. The first index used here is the global feed conversion ratio GFCR (kg/kg) which expresses the whole herd ratio of feed consumption per weight gain. It is a reflection of performance in pig units. The second, the FCR 8–115 (kg/kg), is very similar to GFCR except that it expresses the performance of animal growth from 8 kg to 115 kg, the FCR 8–115 being ever lower than the GFCR since it excludes the sows. Economic management was also evaluated by calculating health costs linked to veterinary bills and treatment.

Fig. 5 shows these indices over an 8-year period which enables the impact of the Din/Pig treatment to be evaluated on the technical-economic management of a model herd. Fig. 5 (top panel) shows that the global health costs are reduced by about 25% over the surveyed study period, essentially due to a sharp decrease in veterinary bills which are mainly for vaccines and antibiotics. The costs associated with the Din/Pig treatment are generally stable which explains the global decrease in the cost related to the animals' health care. Regarding the global feed conversion ratio (GFCR), a continuous decrease was observed for both the global index and for the FCR 8–115, which specifically reflects the performance of pig feeding. They both indicate that pigs are healthier and grow faster with less feed consumed to reach the standard commercial target of 115 kg. The economic balance is therefore further improved in favour of both quality and economic results.

4. Discussion

The economics of pig breeding have become highly critical in a stiff competition between European areas of production. Consequently any conceivable ways of increasing the margin over feed, replacement and health

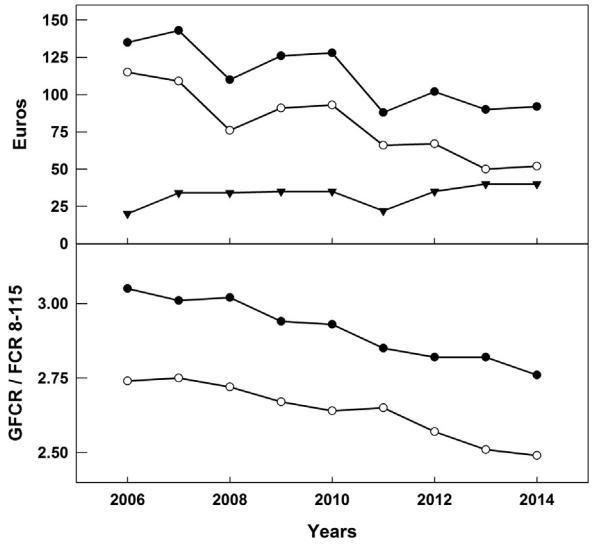

Fig. 5. Evolution in time of health costs and zootechnical parameters. Top panel: Health costs per sow per year. Solid circles, global health costs, open circles, veterinary costs, down triangles, Dinamycol and Pigfast costs. Bottom panel: solid circles, GFCR, open circles, FCR 8–115.

expenses are essential. Factors such as mortality and technical feed conversion ratio are highly critical to the herd's economic balance, along with the associated welfare costs. When facing decreased zootechnical performance and/or digestive malfunction leading to decreased productivity, the herd manager is tempted to turn to antibiotherapy which further increases the phenomena of multi-resistance. To propose a more natural alternative which embraces the whole herd's microbial ecosystem, Dinastim has developed a strategy which aims at improving animal health through the control of biofilm colonisation of the water distribution systems. The present study is focused on assessing the observed beneficial effects of the Din/Pig treatment that were in direct relation with the biofilms present in the drinking water DSs. Even though positive zootechnical effects were observed on few distinct herds, the mechanisms by which both health and the total feed conversion ratio are increased remained undisclosed. The involvement of biofilms present in the drinking water DSs is confirmed by the observation that if the Din/Pig treatment is directly applied to the animals' trough, it results in a much lower benefit. The present study reveals that, subsequent to the Din/Pig treatment, an initial microbial ecosystem – essentially dominated by fungi – is progressively overcome by a new biofilm only bacterial in nature. The link between the presence of fungi in DSs and negative consequences for human or animal health is not new. Some studies have previously reported the presence of hyphae in drinking water [14] although most of the microbial contaminants looked for are bacteria and viruses rather than fungi which are "not expected to grow in water". Nevertheless, Doggett [15] has studied the biofilm of a municipal water DS and observed densities of filamentous fungi ranging from 4 to 25.2 CFU/cm^2. Fungi were also isolated from the water of municipal water distribution networks and from hospital plumbing systems [16–18]. More noticeably, filamentous fungi have been reported as biofilm formers [19]. These findings suggest that biofilms in drinking water distribution networks and hospital plumbing systems can occasionally be a reservoir of fungi with pathogenic properties.

The Din/Pig treatment has been used in eight distinct herds in the Brittany production area with positive effects as mentioned above. Hence, essential oils must play a role in the observed transformation between fungi/bacterial biofilms as observed here by laser confocal microscopy. It can be noted that this microbial ecosystem transformation is associated with a rapid (2–3 months) increase in the zootechnical performance. This point is further supported by a recent study [20] which demonstrates the effect of bacterial biofilm on dairy cattle. It was observed that a poor microbiological quality of drinking water might adversely affect feed intake, herd health and productivity. Furthermore, a link between water microbiology and herd health has been proposed [21] which, based on the phylogenetic study, suggests a connection between bacteria present in the drinking water and those colonising the animals' gastrointestinal tract.

The question of the mechanism of essential oil activity may find an answer in the critical point of physicochemical interactions between (a) biotic surfaces and microbial membranes. These interactions depend highly on the respective physicochemical properties such as hydrophobic/hydrophilic or acid/base characters [22], for example, *Escherichia coli* and *Pseudommonas aeruginosa* membranes present a greater hydrophilic character subsequent to a Dinamycol treatment (data not shown). Moreover, Wösten [23] showed the existence of hydrophobins which are proteins only secreted by filamentous fungi and which promote the attachment of hyphae to hydrophobic surfaces. It can therefore be hypothesized that the Din/Pig treatment alters the pipe-fungi hydrophobic interactions and as a consequence promotes the adhesion of a new bacterial consortium whose development is down-regulated by the fungal population. Another possible mechanism which could explain the reversal of microbial competition would be that the altered physicochemical properties of the pipes' surface promote the adhesion of a new bacterial community which could be responsible for a direct competition with regard to fungi. The present laser confocal observations do not seem to support this second hypothesis since it appears that a significant bacterial biofilm arose *after* the fungal biofilm died or completely vanished. Ref. [24] reports many studies on biofilm *Candida albicans* formation and interactions with other *Candida* species or bacteria. In any case, our data show that, subsequent to treatment using essential oils, the fungal biofilm is replaced by a bacterial biofilm. This phenomenon could be explained by the modification of the physicochemical properties (acid/base or hydrophobic/hydrophilic) of water pipe inner surfaces thereby promoting a de novo bacterial adhesion. The eradication of the fungal biofilm may also be due to either the effects of the essential oils [25] or volatile organic compounds produced by bacteria as previously reported [26]. Ultimately, the inversion of the biofilm is of consequence since it significantly improves the herd's health by allowing a positive biofilm to colonize the water

distribution network with associated positive economic effects on the total feed conversion ratio. Such treatments based on natural extracts could therefore provide a valuable alternative to the spread of antibiotic molecules.

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