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Probiotic *Bacillus sp*. environmental strains as a component of improved dishwasher cleaning product

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ABSTRACT

The use of probiotics has become a mainstream application in pharmaceutical, food, cosmetic and cleaning technologies. Probiotic microorganisms seem to be a remedy for the problem of undesired, persistent microbiological contamination or uncontrolled biofilm formation in the automatic household equipments, dedicated to the undirect contact with food. We propose the mixture of commonly widespread and bio-safe environmental probiotic *Bacillus* sp. strains as a component of an improved cleaning product for automatic dishwashers. Our design of probiotic-improved cleaning product combines the chemical detergent formula and probiotic *Bacillus* sp. preparation in two separate pockets of self-soluble cleaning capsule. To pinpoint the validity of an improved probiotic-based cleaning product design, we present a case study of the microflora from the chosen localizations of an old household dishwasher. The swab collecting, followed by bacterial cultivation and MALDI-TOF identification revealed *Enterobacter cloacae* and *Pseudomonas oleovorans* forming the major inner microflora of an old household dishwasher, where a commonly available dishwashing products were used. We applied probiotic *Bacillus* sp. improved cleaning product to a model dishwasher in a conventional cleaning cycle and conducted the before-after trial to identify the inner microflora in chosen localizations. Using standard microbiological Gram-staining method with microscopic

observation, followed by MALDI-TOF identification we confirmed, that *Bacillus* sp. replaced *Acinetobacter* sp., residing formerly model dishwasher filter and water drain. Conducted experiments have shown the potential for use of the chosen probiotic *Bacillus* sp. strains mixture as an addition for encapsulated formula of cleaning product, to provide microbiologically safe and controllable conditions during the conventional dishwasher cleaning cycle.

***Keywords*:** Dishwasher microbiota, MALDI-TOF microorganism identification, *Bacillus*, Probiotic microorganisms, Surface colonization, *Enterobacter cloacae*, *Pseudomonas oleovorans*

1. INTRODUCTION

Automatic dishwashers have become an important part of household equipment in developed countries. They are valued for economical features, like water savings, and for the convenience of replacing the everyday routine of hand washing dishes, cutlery and other cooking accessories. The mechanism of action combines both the high temperature water jets and the aggressive alkaline chemical formulas of cleaning agents, supplied in the form of powders, tablets or capsules, filled with cleaning gels. Typical cleaning agent designed for the use in the dishwasher may contain polycarboxylates, non-ionic detergents, phosphonates, alternatively blend of hydrolysing enzymes and fragrances. Although the dishwashers and specialized cleaning agents are designed to assure conditions for cleaning the inlet, the dishwasher itself may still be a reservoir of unwanted microbiota. As with other food processing environments and industrial facilities, the dishwasher contains an abiotic surface prone to biofilm formation [1,2].

The organic fragments of food remainings and moist conditions are the major causes of bacterial population growth, especially when the dirty inlet is cleaned the day after or stored for a prolonged term, instead immediate cleaning. The unwanted symptoms like bad smell, colored sludge or limescale, which cause technical defects in the dishwasher, may all be the results of biofilm formation that involves bacteria, algae and fungi. The common foodborne pathogens found in biofilms are *Salmonella* sp., *Listeria* sp., *Escherichia coli* and *Staphylococcus* sp. [1]. Yeast-like opportunistic fungi or *Candida* are reported to be found in the swab material collected from the household dishwashers [3]. The different microorganisms found in the biofilm microbiological reservoir are adsorbed on the phase borders (solid/liquid, liquid/liquid, liquid/gas) [2]. This biofilm is additionally stabilized by an extracellular secretion of metabolic by-products from the microorganism populations. Therefore, it can contain polysaccharides, proteins, phospholipids and nucleic acids that make the impurities even more difficult to remove [4], even when adequate cleaning agents dedicated for cleaning inside dishwashers are applied.

Concerning all above, the scope of the presented experimental data was 1) to identify the microflora from the chosen localizations of an old, used dishwasher, in a case study of household equipment, 2) to propose a strategy to implement an environmental-friendly probiotic preparation to a model dishwasher cleaning cycle and further 3) to test the probiotic-improved cleaning product, identifying the inner microflora in a chosen localizations of the model dishwasher in the before-after trial,.

Probiotics have dozens of specialized applications in health, food and cleaning related technologies, with a number of mainstream probiotic-based products. As probiotics are

microorganisms beneficial for humans or animals in the area of unwanted microflora elimination [5], they can stimulate the immunity of humans/animals while eliminating the risk of atopy or by reducing allergic symptoms [6]. Probiotic microorganisms show a positive effect on gastrointestinal tract (GIT) functioning, and for other systems in the body [7], during long-term residence. Interestingly, certain typically environmental bacterial species, like *Bacillus* sp., are also capable of colonizing the GIT [8] and can have numerous biotechnological applications when controllable and bio-safe microflora are required. *Bacillus subtilis* and other *Bacillus* sp. are reported to competitively exclude *Salmonella* or *Clostridium* strains in animals [9,10] and have a protective activity against *Salmonella* infections [11].

Bacillus subtilis treatment has also long term effectiveness, concerning human patients with urinary tract infections [12], while *Lactobacilli* are known for their anti-diarrheal activity during antibiotic therapy [13]. Probiotics from the *Bacillus* genus are proposed as biological control agents in the aquatic environment [14], whereas *Bacillus subtilis* and *Bacillus pumilus* serve as surface biocontrol components for hospital-dedicated cleaning products [15]. Concerning all of the above, our presented strategy for unwanted microflora replacement in dishwashers is to apply the preparation of environmentally-friendly probiotic microorganisms from the *Bacillus* genus in the improved dishwasher cleaning product.

2. MATERIALS AND METHODS

2. 1. Microorganisms cultivation and microscoping

The soy peptone for microbiological media preparation was from Scharlau Microbiology (Barcelona, Spain), other reagents like yeast extract, sodium chloride, bacteriological agar and dyes for bacterial-cell staining were from Sigma-Aldrich (St Louis, MO, USA). Microorganisms cultivation and plate streaking were performed using standard microbiology methods [16]. All incubations were made in a New Brunswick Scientific (Boulevard, CT, USA) or Binder (Tuttlingen, Germany) microbiology incubators. The microbiological properties were determined using the Gram-staining method [17], slide glass preparations were observed under an Olympus CX21FS1 light microscope with total 1200x magnification.

2. 2. The old household dishwasher microflora identification – a case study.

The old dishwasher microflora identification was performed in approx. 10 years old Bosch dishwasher, where the conventional cleaning products were used. Sterile swab sticks (BIONOVO, Poland) were used for collecting swab samples from dishwasher filter and the water drain hose. The swabs were plated on LA medium, followed by 24 h, 37 ºC incubation.

The MALDI-TOF method [18,19] was used for the bacterial species identification. The swab microorganisms cultures were pooled from the agar plates and diluted in LB medium, then plated onto LA medium [20] to obtain single colonies on LA medium. The single colonies were isolated and subjected to a MALDI Biotyper (Bruker Daltonics, Billerica, MA, USA) at Laboratoria Medyczne Bruss (Gdynia, Poland). The resulting spectra were compared with intracellular protein profile databases for microbiological species (not shown).

2. 3. The model dishwasher microflora identification – a before-after trial with probiotic *Bacillus* sp. improved cleaning product

The microflora identification in a before-after trial, while using the probiotic-improved cleaning product, was performed in the model dishwasher Bosch, type SMS50D38EU. Swab collecting, microorganisms cultivation on LA agar, microscoping and MALDI-TOF identification were performed as described above.

The constructed probiotic-improved dishwasher cleaning product contained non-ionic detergents, polycarboxylates, phosphonates and a blend of enzymes in self-soluble capsule with an addition of 20% portion of probiotic preparation, closed in a separate pocket of the same capsule [21] The probiotic preparation chosen for the study was the environmental *Bacillus* sp. commercial mixture Bacilox® (Osprey Biotechnics, Sarasota, FL, USA), with dominating *Bacillus subtilis*.

The swabs were collected before and after passing the automatic 3 hours cleaning program, involving pre-cleaning, 50 °C cleaning, pre-rinsing and 65 °C rinsing, followed by drying. The dirt inlet was placed in the dishwasher, in order to reproduce the complex conditions of the household equipment. The prepared dirt consisted of model dishes, covered with oat-milk, preheated egg yolks and a portion of fat, protein and sugar mixture.

3. RESULTS AND DISCUSSION

To point out the validity of probiotic enriched dishwasher cleaning strategy, we conducted a case study experiment, to identify the inner microflora of an old dishwasher, where the commonly available dishwasher cleaning products were used for over 10 years. The sources for swab collecting in the old dishwasher, as well as in the model one in subsequent experiments, were dishwasher water drain hoses and water filters. The localizations for microorganism sampling were intentionally chosen, as the most critical parts collecting dirt and food remainings (filter) or having constant contact with water (filter, drain hose). These points of the dishwasher assure conditions, required for microorganisms proliferation, therefore are the most difficult to control. The swabs were taken from the chosen localizations in an approx. 10 year old, household dishwasher, followed by microbiological streaking on LA plates, which resulted in massive bacterial growth at 37 °C, as shown in Fig. 1 (a and b).

Subsequently, the bacterial cells mass was pooled from agar medium, diluted in liquid medium LB, dilutions plated again to obtain well separated, single colonies suitable for MALDI-TOF identification. The chosen MALDI-TOF microorganism identification method is based on total protein mass spectrum comparison to a database of various microorganisms [18,19]. The method allows for the reliable and rapid identification to the species level, being comparable to serotyping or nucleic acid mediated methods, while typically more accurate than biochemical methods [19,22]. An identification index value was given to assess the accuracy of the result. The obtained data allowed for the reliable identification to the genus level, while probable identification to the species level of *Enterobacter cloacae* and *Pseudomonas oleovorans*, with identification indexes between 2.30 – 3.00. (Table 1a). *Pseudomonas oleovorans* is a methylotrophic bacteria [16], residing in cooling agents or lubricants, while *Enterobacter cloacae* [16] can be a human physiological GIT microflora, although it can cause potential health risk, thus being an unwanted contamination.

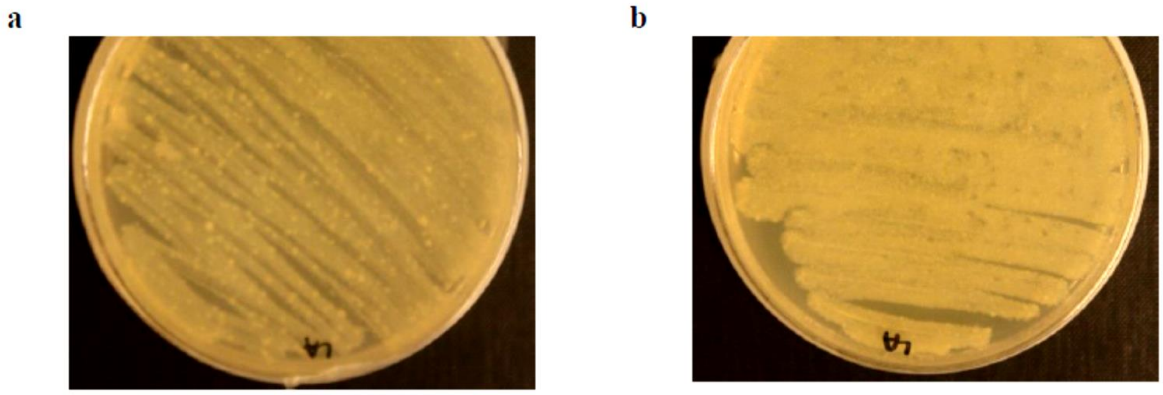

Figure 1. Growth of microorganisms from an old dishwasher swabs (a case study). Petri dishes with LA medium, after 24 hours incubation at 37 ºC. Source of swab samples for performed streaks: a - Filter, b - water drain.

Table 1. Dishwasher microflora identification obtained using the MALDI-TOF method.

a. Old dishwasher inner microflora identification. A case study with conventional cleaning product		
Dishwasher part	Predominant bacterial species	ID*
Water drain	*Enterobacter cloacae*	2.276
Filter	*Pseudomonas oleovorans*	2.294
b. Model dishwasher inner microflora identification. A before-after trial with probiotic *Bacillus* sp. improved cleaning product		
Dishwasher part	Predominant bacterial species	ID*
Water drain before cleaning	*Acinetobacter haemolyticus*	2.133
after cleaning	*Bacillus subtilis*	1.824
Filter before cleaning	*Acinetobacter haemolyticus*	2.237
after cleaning	*Bacillus subtilis*	1.978

*ID - the MALDI-TOF species identification index value spans (according to Azarko et al., 2011): 2.30 – 3.00 reliable identification to the species level; 2.00 – 2.299 reliable identification to the genus level and probable identification to the species level; 1.70 – 1.999 probable identification to the genus level; 0 – 1.699 unreliable identification.

We designed and constructed the probiotic-improved dishwasher cleaning product, closed in the self-soluble capsule, with the separate pocket filled with a portion of lyophilized *Bacillus* sp. preparation (Fig. 2). We chose the mixture of environmental *Bacillus* sp. strains, present in the commercial preparation Bacilox®, for the component of the improved cleaning product. The bacterial preparation contains several *Bacillus* probiotic species, with *Bacillus subtilis*, as we characterized elsewhere, using the MALDI-TOF method [18,22]. The bio-safe environmental *Bacillus* sp. strains, including *Bacillus subtilis*, are capable of producing spores and possess an increased environmental stability and permeability [23]. Their survivability covers ecological niches of vast temperatures and pH values.

Figure 2. Lyophilized probiotic *Bacillus* sp. environmental strains as a component of improved dishwasher cleaning product in the two-pockets self-soluble capsule.

By the mean of the constructed cleaning capsule we implemented a portion of probiotic preparation, during the cycle of cleaning with an artificial dirty inlet, in the model dishwasher. The microscope imaging of Gram-stained bacterial population, collected from the model dishwasher is shown in Fig. 2 (a and b). It represents a pool of microorganisms collected from the water drain before the cleaning cycle (Fig. 2a) and directly after cleaning with *Bacillus* sp. probiotic supplementation (Fig. 2b).

The observed dominating morphology of Gram–minus cocci in the samples collected before cleaning, were identified subsequently as *Acinetobacter haemolyticus*, using the MALDI-TOF method. The identification index value pointed the result is reliable to the genus or even species level (Table 1b). However, in the sample collected from the dishwasher water drain directly after the cleaning with the probiotic-improved cleaning product, the Gram-staining method revealed Gram–positive, rod-shaped cells, which MALDI-TOF detected as *Bacillus* sp. (*Bacillus subtilis*), with an identification index on the probable genus identification level. The precise identification of *Bacillus* sp. to the species level was problematic, as the analysed species were present in a mixture of very closely related environmental strains [23]. The *Bacillus* sp. was also identified in the swabs collected from

the dishwasher filter, after cleaning with the probiotic-improved cleaning product. The implemented probiotic species successfully replaced the formerly residing *Acinetobacter*, as MALDI-TOF confirmed (Table 1b).

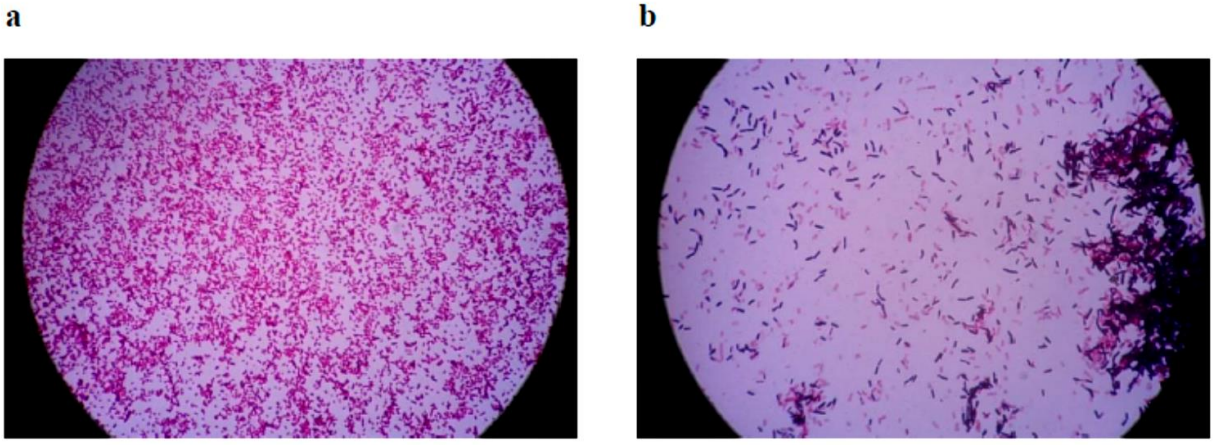

Figure 2. Microscope imaging of Gram-stained bacterial population, collected from the model dishwasher water drain: a - before cleaning cycle, with dominating *Acinetobacter haemolyticus*, as subsequently identified by the MALDI-TOF method; b - after a cleaning cycle with the addition of *Bacillus* sp. probiotic preparation, with dominating *Bacillus subtilis*, as subsequently identified by the MALDI-TOF method. Cleaning cycle performed with the use of improved dishwasher cleaning product, containing lyophilized probiotic *Bacillus* sp. environmental strains.

4. CONCLUSIONS

In the presented study we pointed the household dishwasher may be a source of an unwanted microbiota. We proposed an improved, probiotic *Bacillus* sp.-based cleaning product, which successfully introduced an environmental bio-safe microflora into the dishwasher. The probiotic improved dishwashing cleaning product is of dual significance: as a biological treatment for unwanted microbiota and as a possible technological remedy for the problem of biofilm formation, containing various and changeable microorganisms on the dishwasher parts.

The major conclusions of our study are as follows:

1. Dishwashers constructed for automatic cleaning of food remains can also be the reservoirs of an unwanted microbiota, causing a health risk or forming biofilms that may deteriorate the cleaning effectiveness and durability of dishwasher construction parts.

2. Combining microbiological methods with subsequent MALDI-TOF mass spectrometry microorganisms identification, we determined in a case study that the bacterial species *Enterobacter cloacae* and *Pseudomonas oleovorans* form the major inner microflora of the vulnerable parts of an old dishwasher filter and water drain.

3. We proposed the advantage of using the probiotic *Bacillus* sp. mixture in the improved, probiotic-based cleaning product dedicated for dishwashers.

4. We successfully applied a probiotic preparation into the model dishwasher operation, during a conventional cleaning cycle. The bacterial identification, using standard microbiological Gram-staining method with microscopic observation, followed by MALDI-TOF identification confirmed, that *Bacillus* sp. replaced *Acinetobacter* sp., residing formerly dishwasher filter and water drain.

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