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Method for Bacterial Growth and Ammonia Production and Effect of Inhibitory Substances in Disposable Absorbent Hygiene Products

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ABSTRACT

PURPOSE: The purpose of this study was to evaluate a pragmatic laboratory method to provide a technique for developing incontinence products better able to reduce malodor when used in the clinical setting.

METHODS: Bacterial growth and bacterially formed ammonia in disposable absorbent incontinence products was measured by adding synthetic urine inoculated with bacteria to test samples cut from the crotch area of the product. The inhibitory effect's of low pH (4.5 and 4.9) and 3 antimicrobial substances—chlorhexidine, polyhexamethylene biguanide (PHMB), and thymol—at 2 concentrations each, were studied.

RESULTS: From the initial inocula of 3.3 log colony-forming units per milliliter (cfu/mL) at baseline, the bacterial growth of the references increased to 5.0 to 6.0 log cfu/mL at 6 hours for *Escherichia coli*, *Proteus mirabilis,* and *Enterococcus faecalis*. At 12 hours there was a further increase to 7.0 to 8.9 log cfu/mL. Adjusting the pH of the superabsorbent in the incontinence product from 6.0 to pH 4.5 and pH 4.9 significantly ($P < .05$) inhibited the bacterial growth rates, in most cases, both at 6 and 12 hours. The effect was most pronounced at pH 4.5. Chlorhexidine had significant ($P < .05$) inhibitory effect on *E. coli* and *E. faecalis*, and at 12 hours also on *P. mirabilis*. For PHMB and thymol the results varied. At 6 hours, the ammonia concentration in the references (pH 6.0) was 200 to 300 ppm and it was 1500 to 1600 ppm at 8 hours. At pH 4.5, no or little ammonia production was measured at 6 and 8 hours. At pH 4.9, there was a significant reduction ($P < .01$). Chlorhexidine and PHMB exerted a significant ($P < .01$ or $P < .001$) inhibitory effect on ammonia production at both concentrations and at 6 and 8 hours. Thymol 0.003% and 0.03% showed inhibitory effect at both 6 hours ($P < .01$ or $P < .001$) and at 8 hours ($P < .05$ or $P < .001$).

CONCLUSION: The method described in this study can be used to compare the ability of various disposable absorbent products to inhibit bacterial growth and ammonia production. This technique, we describe, provides a pragmatic method for assessing the odor-inhibiting capacity of specific incontinence products.

KEY WORDS: Absorbent hygiene products, Ammonia production, Bacterial growth, Diapers adult, Incontinence, Incontinence pads, Odor control.

INTRODUCTION

Disposable absorbent hygiene products are widely used for containment of urinary and fecal incontinence. The main purpose of such products is to hold urine or fecal materials without leaking. Other important characteristics are the ability to control odor, to stay in place, discreteness, comfort when wet, and to keep the skin dry.[1] In an overview on research priorities to improve incontinence products, Fader and colleagues[2] point to techniques and designs from basic laboratory science, to clinical trials of products and to evaluations of service delivery models. Furthermore, research includes determining prevalence of cost and product use, development of patient-reported outcomes, and development of methods for measuring skin health and for quantifying urine or fecal leakage.[2]

Multiple factors are postulated to contribute to the odor produced by used incontinence products, including volatile chemicals within the urine itself or products of bacterial metabolism.[3] Bacteriuria is prevalent in a small portion of the general population and it is more prevalent in persons suffering from urinary incontinence.[4] During their use, the incontinence products will come in close contact with the perigenital and perineal skin, which contains different biological materials as well as microflora. Volatile compounds may be generated by the bacterial metabolism of different substances and by different chemical and enzymatic reactions. The formation of ammonia from urea by bacterial ureases, such as *Proteus*, has been considered a key component of odor in patients with urinary incontinence.[5]

The problem with odor in urine has been addressed in several publications.[6,7] Common bacteria, such as *Escherichia coli*, *Proteus mirabilis,* and *Enterococcus faecalis*, have been reported to generate a number of volatile odorous compounds after incubation of urine.[6,7] Development of incontinence products has focused not only on absorption of urine but also on

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substances that can inhibit bacterial activity and thus reduce the development of foul smells. For example, the addition of copper acetate to the product was an early attempt to reduce the disagreeable odor associated with ammonia.[5]

To the best of our knowledge, no article has been published on methods to control bacterial growth and ammonia production in disposable absorbent products. The recent work of Sironi and colleagues[8,9] describes a method for sensory evaluation of odor in incontinence products but does not describe techniques for controlling these odors. To control malodors associated with wearing absorbent products, it is necessary to develop methods to measure odor, bacterial load, and ammonia production. Laboratory methods are often a simplification of a complex clinical setting; they should be robust and reproducible. Because human urine is highly variable in its composition,[10] standardizing content via use of a defined synthetic urine is desirable. The purpose of this study was to test a method for measuring bacterial growth and ammonia production in disposable absorbent hygiene products. Common antimicrobial substances are tested to illustrate application of the method.

MATERIALS AND METHODS

We measured bacterial growth and bacterially formed ammonia in disposable absorbent incontinence products by adding synthetic urine inoculated with bacteria to test samples cut from the crotch area of the product. *E. coli* CCUG 24 (http://www.ccug.gu.se), *P. mirabilis* CCUG 4637, and *E. faecalis* CCUG 9997 were stored in cryotubes at −20°C until use. One pearl of each bacterial strain was applied to Trypticase Soy agar plates (Oxoid LTD, Basingstoke, England), which were incubated at 30°C overnight (16-18 hours). An inoculum was then transferred into nutrient broth tubes (NB Oxoid CM1) and cultured at 30°C for 16 hours. For the *E. faecalis* strain, the nutrient broth was supplemented with 1% glucose for better growth. All 3 bacterial strains were originally human urinary isolates.

Preparation of Synthetic Urine

A stock solution was prepared containing 0.15M sodium chloride (Scharlau, Sentmenat, Spain), 0.02M dipotassium hydrogen phosphate (Merck KGaA, Darmstadt, Germany), 0.01M sodium dihydrogen phosphate (dihydrate) (VWR BHD Prolabo, Leuven, Belgium), 0.05M ammonium chloride (Merck), 0.02M disodium sulphate (decahydrate) (Merck), 0.005M lactic acid (90%, Prolabo), yeast extract (Becton Dickinson, Sparks, MD), and distilled water up to 1000 mL. The stock solution was sterilized by autoclaving. A urea/glucose solution was prepared containing 6M urea (Scharlau), 0.01M D-glucose (Prolabo), and distilled water up to 100 mL. This solution was sterilized by filtration through 0.22-μm filter. A cationic solution containing 0.3M magnesium chloride (hexahydrate) (Merck), 0.3M calcium chloride (dihydrate) (Merck), and distilled water up to 20 mL was sterilized by autoclaving. Each solution was kept at +4°C, until mixing. Synthetic urine (SU) was prepared by mixing a 94-mL stock solution with 5-mL urea/glucose solution, and 1-mL cationic solution. The readymade solution (pH 6.6 ± 0.1) was kept at +4°C and used within a week of preparation.

Absorbent Product Test Samples

Incontinence products are composed of a water-permeable top sheet of nonwoven material, a waterproof back sheet of plastic film, and in between is an absorbent core consisting of a mix of cellulose pulp and superabsorbent polymer (SAP). Test samples were cut (tool diameter 5 cm) from the crotch area of an incontinence product.

A bacteria inhibitor was added to each sample. To illustrate the method and the inhibitory effect, the following antibacterial substances were selected: chlorhexidine (Alfa Aesar GmbH & Co Kg, Karlsruhe, Germany), PHMB (polyhexamethylene biguanide) (Arch UK Biocides, Castleford, UK), and thymol (Acros Organics, Morris Plains, Morris County, New Jersey). Two different concentrations of each substance were used as determined in pretests: chlorhexidine 1% and 0.1% (vol%/mL SU), PHMB 5% and 0.5% (wt% on pulp), and thymol 0.03% and 0.003% (vol%/mL SU). Solutions of the substances were evenly distributed on the test samples.

The inhibitory effect of low pH was tested in samples with pH 4.5 and 4.9 of the SAP (BASF, Ludwigshafen, Germany). All test samples were compared to a reference with pH 6.0 of the SAP.

We calculated a theoretical volume of urine (V mL) based on the amount of synthetic urine that can be absorbed in the absorbent core. This volume also corresponded to the urine absorbed in the wet area of a product in use.

Bacterial Growth

The nutrient broth cultures were diluted in synthetic urine and mixed in equal proportions to a final mixture containing 3.3 ± 0.3 log colony-forming units (cfu) of each microorganism per mL urine test medium. Synthetic urine with microorganisms was added to sterile plastic jars (UniPak 120 mL, RPC Superfos, Taastrup, Denmark) in triplicates in predetermined volumes as described earlier. Each test sample was gently placed upside down in the jar and left for 5 minutes to evenly absorb the urine test medium. The jar was closed and turned upside down and incubated at 35°C (±0.5°C). Samples for growth determination were taken after 6 and 12 hours. The number of viable bacteria was counted after each test sample was transferred to a sterile plastic Stomacher bag (specially designed for storing solutions subjected to blending or homogenization) that was filled with peptone water (0.85% NaCl [Scharlau] + 0.1% peptone [BD]), diluted 1:10 in relation to the amount of SU. The test sample was homogenized in the Stomacher bag for 3 minutes to release the bacteria from the absorbent product. The homogenate was serial diluted, cultured on agar plates, and incubated aerobically at 30°C for 24 to 48 hours.

E. coli and *P. mirabilis* were cultured on modified Drigalski agar plates (Clinical Microbiology, Sahlgrenska University Hospital, Gothenburg, Sweden), and *E. faecalis* was cultured on Slanetz Bartley plates (Oxoid CM377, Swedish Institute for Food and Biotechnology, Gothenburg, Sweden). The number of cfu per mL urine test medium was then calculated.

Ammonia Production During Bacterial Growth

Two hundred milliliters of SU was inoculated with the *P. mirabilis* culture and incubated in a shaking apparatus (IKA OS 5, IKA-Werke GmbH & Co KG, Staufen, Germany, 240 opm) at 35°C. The size of the inoculum and the incubation time were adjusted to achieve a final bacterial concentration of 4.0±0.5 log cfu/mL of synthetic urine. The bacterial solution was diluted 1:5 with SU warmed to 35°C. Each test sample was placed in sterile plastic jars (240 mL with a snap-on lid, Kemikalia, Skurup, Sweden). Before use, a hole (diameter 7.25 mm) was drilled in each lid. A silicone tube (length 5 cm,

outer diameter 8 mm) was fed into the hole and then the jar was autoclaved. The tube was sealed with a cork. Synthetic urine with microorganisms was added to the test sample in the volume determined by the volume of synthetic urine. The jar was closed and incubated at 35°C. Six replicates of each test sample were prepared.

Samples for ammonia concentration were taken after 6 and 8 hours using Dräger gas detector tubes for ammonia (http://www.draeger.com). A calibrated 100-mL sample of air was drawn through a tube (a glass vial filled with a reagent that reacts to specific chemicals) using a Dräger accuro Bellows Pump. The length of the color change indicates the concentration measured. Tubes for ammonia with ranges measuring 5 to 100 ppm or 5 to 600 ppm were used. For values above 600 ppm, a syringe was used instead of the Dräger pump in order to extract a smaller sample of 50 or 25 mL. The results were multiplied by 2 or 4. Before the measurements were done, the jar was punctured 1 cm above the bottom with a 0.6-mm injection needle to prevent a vacuum. The end of the Dräger tube was broken by using a pipe cutter. The cork was removed and the tube inserted 0.5 cm into the silicone tube.

Data Analysis

Bacterial Growth

An á priori calculation gave 80% power of detecting 1 log cfu/mL reduction for the sample size of 3 in a 2-way analysis of variance (ANOVA). The bacteria amount, measured as log cfu/mL, was defined as the outcome variable. For bacterial counts below the detection limit (2 log cfu/mL [or occasionally 3 log cfu/mL]), there are no standard statistical methods; therefore, random values from a uniform distribution in the log interval 0 to 2 (or 0-3) were generated. A 2-way ANOVA, with treatment (inhibitor) and time as factors and their interaction, was used to analyze outcomes. Adjustments for multiple testing were done using the Holm-Bonferroni method.[11]

Ammonia Production

An á priori calculation gave 80% power of detecting a 40% reduction for the sample size of 6. The ratios of inhibitor and reference measurements were computed within each synthetic urine batch. The geometric mean values of ratios gave estimates of the percentages of the remaining contents of ammonia products versus references. Pairwise *t* tests were applied to the log ratios. Bonferroni's correction was applied to the nominal significance level of .05 amounting to a "*critical *P* value" of .00555 for the antimicrobial substances and to .01667 for the pH samples.

RESULTS

We first measured bacterial growth in the reference inocula. This analysis revealed that the initial inocula of 3.3 log cfu/mL at time 0 hour increased bacterial growth to 5.0 to 6.0 log cfu/mL at 6 hours for the 3 bacterial strains. At 12 hours, the corresponding numbers were 7.0 to 8.9 log cfu/mL (Figures 1 and 2, reference pH 6.0). This showed that when no inhibitors were present the test system allowed growth over time.

Adjusting the pH of the SAP in the incontinence product to pH 4.5 or pH 4.9 significantly inhibited the bacterial growth rates compared to the reference, both at 6 and 12 hours, in most cases (Figure 1). The effect was most pronounced at pH 4.5.

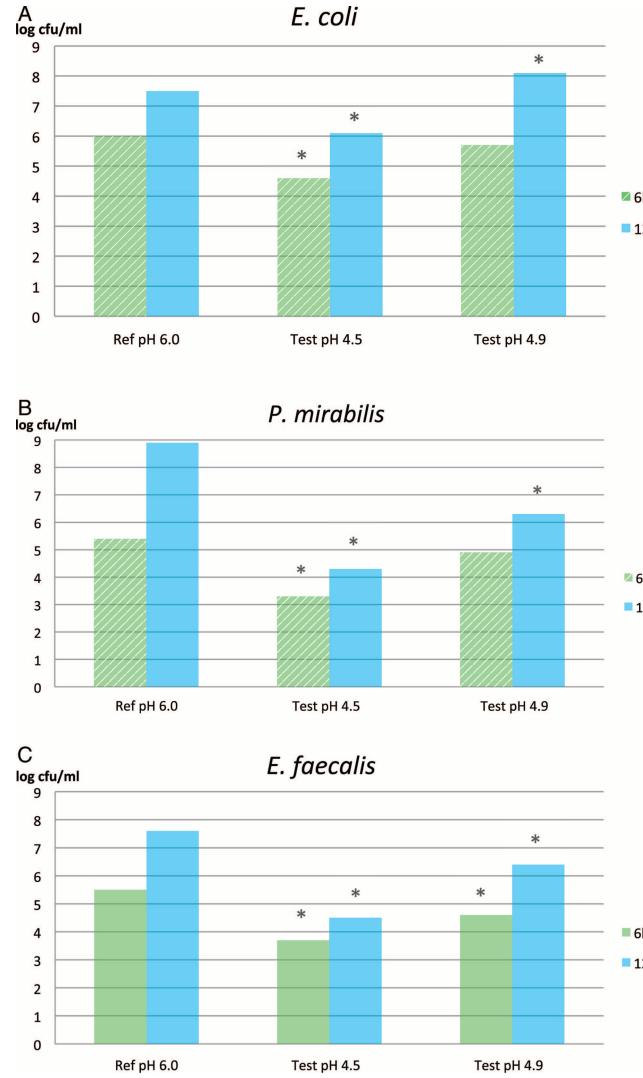

Figure 1. (A). Inhibitory effect of low pH on bacterial growth for *E. coli* after 6 and 12 hours. Columns represent mean values. *$P < .05$. (B). Inhibitory effect of low pH on bacterial growth for *P. mirabilis* after 6 and 12 hours. Columns represent mean values. *$P < .05$. (C). Inhibitory effect of low pH on bacterial growth for *E. faecalis* after 6 (green columns) and 12 hours (blue columns). Columns represent mean values. *$P < .05$.

A comparison to the reference showed that chlorhexidine had a significant inhibitory effect at both concentrations on *E. coli* and *E. faecalis* (Figures 2A and 2C). In contrast, inhibition of the growth of *P. mirabilis* was not significant until 12 hours (Figure 2B). PHMB showed a significant antibacterial effect for *E. coli* and *E. faecalis* at the higher concentration, whereas *P. mirabilis* was unaffected. Thymol 0.03% inhibited the 3 bacteria at 12 hours. No other effect of thymol was seen.

Ammonia Production

At 6 hours, the ammonia concentration in the references was 200 to 300 parts per million (ppm) and it was 1500 to 1600 ppm at 8 hours (Figures 3 and 4). This finding indicates that the test system we designed also allowed ammonia production over time when no inhibitors were present.

When the pH of the SAP was 4.5, little or no (≤5 ppm) ammonia production occurred at 6 and 8 hours (Figure 3). In contrast, when the pH of the SAP was 4.9, we observed

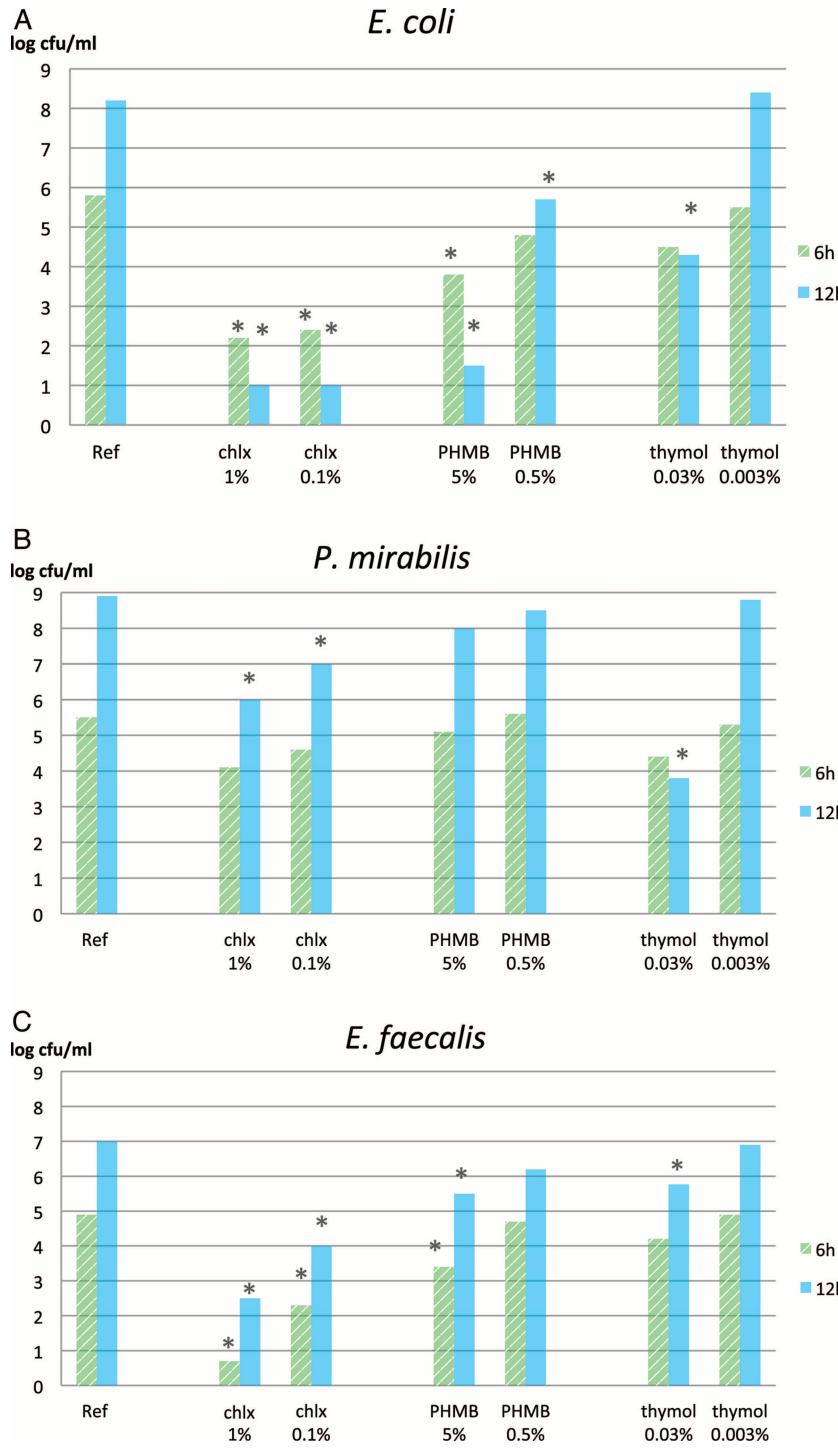

Figure 2. (A). Inhibitory effect of chlorhexidine, PHMB, and thymol on bacterial growth for *E. coli* after 6 and 12 hours. *$P < .05$. (B). Inhibitory effect of chlorhexidine, PHMB, and thymol on bacterial growth for *P. mirabilis* after 6 and 12 hours. *$P < .05$. (C). Inhibitory effect of chlorhexidine, PHMB, and thymol on bacterial growth for *E. faecalis* after 6 and 12 hours. *$P < .05$.

significant reduction in ammonia production, compared to the reference, at both 6 and 8 hours. Chlorhexidine, PHMB, and thymol each had inhibitory effect on ammonia production for both concentrations at 6 and 8 hours (Figure 4).

DISCUSSION

We evaluated a pragmatic laboratory method for bacterial growth and formation of ammonia in disposable absorbent products and we tested the inhibitory effects of low pH and 3 antibacterial substances on these outcomes. Test results show that the growth of bacteria and the formation of ammonia can be simulated as a tool to evaluate the ability of possible antimicrobial substances to control odor in disposable absorbent incontinence products. Ammonia was used as the indicator for any odors in connection to the use of incontinence products. Although important, it should be noted that ammonia production is not the only cause of odor in urine.[3]

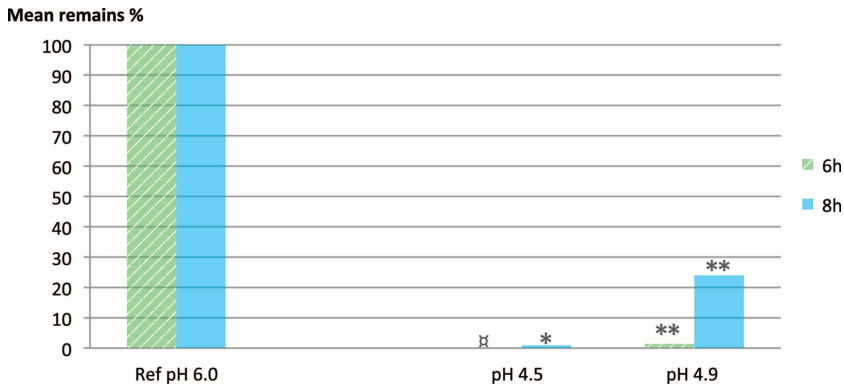

Figure 3. Inhibitory effect of low pH on ammonia production for *P. mirabilis* after 6 and 8 hours. *$P < .05$; **$P < .01$. ¤Geometric mean not computed as test samples contained no ammonia.

Bacterial growth and ammonia production were measured at arbitrarily defined points of time because continuous measurement based on kinetics would have been too cumbersome. The kinetic curve of ammonia production was more rapid than the bacterial growth curve (data not shown); therefore, we chose to measure ammonia production at 6 and 8 hours versus measurement of bacterial growth at 6 and 12 hours. The somewhat different starting inocula, 3.3 and 4.0 log cfu/mL, respectively, for bacterial growth and ammonia production were also selected for practical reasons.

We further evaluated the method used in this study by selecting inhibitors with known antimicrobial activity. A low pH is a well-known method for inhibiting bacterial growth in food preservation, cosmetics, and various hygiene products. Chlorhexidine is a broad-spectrum antimicrobial substance widely used in the health care sector. PHMB has broad antimicrobial activity; it is used as a cleansing agent in swimming pools.[12] Thymol has antimicrobial activity because of its phenolic structure.[13]

Incorporation of an antimicrobial substance in absorptive incontinence products must balance antimicrobial activity and potential adverse side effects on the skin. Maintaining this balance is based on avoidance of known contact allergens, avoiding direct contact with the skin, and careful attention to the concentration of the antimicrobial substance. Thymol and chlorhexidine seem to be less likely candidates for use in incontinence products since they have been reported to irritate the skin and to cause allergic reactions.[14,15] An acidic pH in the range, which was tested here, is well in accordance with normal cutaneous pH values reported in literature.[16] A reduced pH also may be beneficial since it has been demonstrated that skin with low pH values (<5.0) has stronger barrier function.[16]

The reference columns (Figures 1 and 2) show that after the initial inocula of approximately 3 log cfu/mL, bacterial numbers at 6 hours had increased another 3 log steps, with a further increase at 12 hours. In contrast, the antibacterial substances and low pH inhibited or delayed the bacterial growth. The degree of inhibition probably reflected the concentrations of inhibitors chosen for the experiment. For *E. coli*, treatment with chlorhexidine seemed to kill the bacteria since the 12-hour value was lower than the 6-hour value. This was also the case for PHMB 5%. In all other cases, the 12-hour values were higher than the 6-hour values, indicating a partial inhibitory effect of the antibacterial substance or the pH. Figure 1A shows that the *E. coli* growth was highest in the pH 4.9 group at 12 hours. This might be attributable to an increased pH (8.8-8.9, data not shown) caused by growth and activity of *P. mirabilis* decomposing urea to ammonia. Such a high pH slows the growth or decreases the number of *E. coli* cells.

The *P. mirabilis* strain was the only urease-producing strain we selected; it was purposely used in the ammonia production experiment. The ammonia produced in the references reached a level of 200 to 300 ppm after 6 hours and 1500 to 1600 ppm after 8 hours, corresponding to the 100% remaining in Figures 3 and 4. This finding further demonstrates the validity of the method we evaluated. Inhibition or ammonia production may have been influenced by the addition of antimicrobial substances or the low pH. In particular, the combination of a lower pH (4.5) and chlorhexidine showed a great inhibitory effect (Figures 3 and 4).

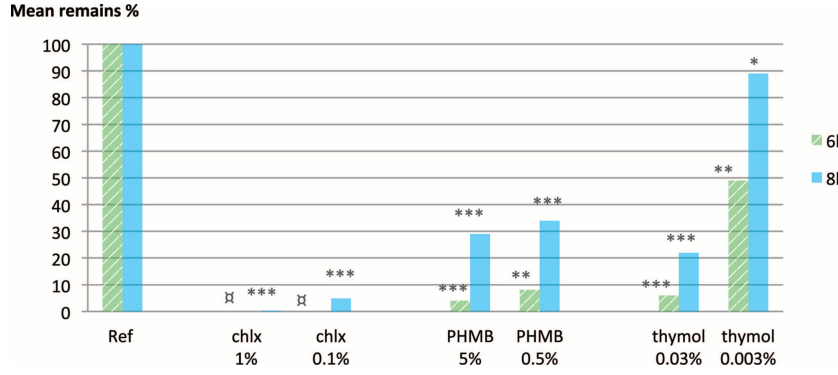

Figure 4. Inhibitory effect of chlorhexidine, PHMB, and thymol on ammonia production of *P. mirabilis* after 6 and 8 hours. *$P < .05$; **$P < .01$; ***$P < .001$. ¤Geometric mean not computed as test samples contained no ammonia.

To allow for statistical analyses, triplicates were done for bacterial growth and 6 replicates for ammonia determination. One problem was how to denote the detection limit for growth (eg, <2 log cfu/mL). This is a common way of denoting low numbers for a microbiologist, but posed a problem in the statistical analyses. The solution was to randomize for low values below the detection limit.

The method for determining bacterial growth with or without inhibitors was shown to be stable and reliable and give reproducible results. On the other hand, the method for assessing ammonia production showed more varied results, which can partly be explained by the Dräger technique and variations in biological activities. However, the simplicity of these tubes is appealing.

The disposable absorbent hygiene products available on the market today were developed during the last few decades, and the search to develop better products to minimize the discomfort of leakage and odor is ongoing. The method described in this article makes a contribution to assessing the inhibitory effect of various substances on bacterial growth and ammonia production in order to reduce odor. Our group (Ref 17-18) has further investigated various aspects on odor. Using standardized methods can facilitate the systematic work of improving disposable absorbent hygiene products, all for the better care and improved quality of life of the many individuals suffering from incontinence. The method described in this article also may contribute to development of a standard for measuring odor control when comparing disposable absorbent hygiene products.

Limitations

Evaluation of urine odor via a standardized synthetic urine formulation, such as the one we developed, is a simplification of the variable and complex factors present in human urine contained in an absorptive product. Similarly, we selected 3 common uropathogens—*E. coli*, *P. mirabilis,* and *E. faecalis*—to represent the complex aerobic and anaerobic bacterial flora of the perigenital and perineal skin. The composition of the synthetic urine solution we evaluated was based on the metabolic requirements for *E. coli* and common concentrations of chemical compounds in human urine.[19] Nevertheless, the laboratory method we describe permits screening for numerous antimicrobial substances and concentrations and has the potential to allow testing of the most promising candidates before most costly and laborious consumer tests are completed.

CONCLUSION

We described a pragmatic laboratory-based method for determining bacterial growth and formation of ammonia. This method can be used to compare the ability of disposable absorbent hygiene products to inhibit growth and ammonia production. It is intended to provide a technique for developing incontinence products better able to reduce malodor when used in the clinical setting.

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