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***Staphylococcus aureus* toxic shock syndrome toxin-1 (TSST-1) production and**
***Lactobacillus* species growth in a defined medium simulating vaginal secretions**

Robin L. Stingley^{a§}, Huanli Liu^{b§}, Lisa B. Mullis^c, Christopher A. Elkins^d, and Mark E. Hart^{c, e*}

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ABSTRACT

Lactobacillus species are commensal with the healthy vaginal environment and inhibit the growth of many pathogenic bacteria in the vaginal tract by a variety of mechanisms, such as the production of hydrogen peroxide, organic acids, and antimicrobial substances. Simulation of the vaginal environment is crucial for proper investigation of the effects of *Lactobacillus* species on pathogenic bacteria. In this study, we modified a medium used to simulate vaginal secretions to improve the growth of toxic shock syndrome toxin-1 (TSST-1)-producing *Staphylococcus aureus* clinical strains and *Lactobacillus* species so that interactions between these bacteria may be examined. A medium consisting of basal salts, vitamins, albumin, glycogen, mucin, urea, sodium bicarbonate, polyoxyethylene sorbitan monolaurate, and amino acids supported the growth of *S. aureus* and the production of TSST-1 as determined by western analysis. Improved growth of the *Lactobacillus* species was seen when this same medium was supplemented with manganese chloride, sodium acetate, and an increase in glucose concentration. However, growth of *S. aureus* in the supplemented medium resulted in reduced levels of TSST-1. Production of TSST-1 was not detected in a medium routinely used for the growth of *Lactobacillus* species although *S. aureus* growth was not inhibited. The development of an improved genital tract secretions medium provides a more authentic environment in which to study the interactions of *Lactobacillus* species and vaginal pathogens, such as *S. aureus*.

1. Introduction

The vaginal ecosystem is a complex environment populated by a variety of commensal bacteria (Bartlett et al., 1978; Bartlett et al., 1977; Ma et al., 2012; Onderdonk et al., 1977; Ravel et al., 2011; Zhou et al., 2010; Zhou et al., 2004), and subject to infection by a number of pathogenic bacteria (Hammill, 1989, Issa and Thompson, 2001). The most prevalent bacteria among the vaginal microbiota are *Lactobacillus* species (Bartlett et al., 1977; Ma et al., 2012; Onderdonk et al., 1977; Ravel et al., 2011; Zhou et al., 2010; Zhou et al., 2004). *Lactobacillus* species provide a measure of protection from colonization of the vaginal vault by pathogenic bacteria using a number of mechanisms. *Lactobacillus* species produce organic acids, hydrogen peroxide and bacteriocins, which act individually or in combination to inhibit the growth of pathogens and, in some cases, their production of specific factors (Aroutcheva et al., 2001, Aslim and Kilic, 2006, Dembélé et al., 1998, Elkins et al., 2008, Laughton et al., 2006, Ocana et al., 1999, Osset et al., 2001, Otero and Nader-Macias, 2006, Pascual et al., 2006, Vesterlund et al., 2006). In addition, *Lactobacillus* species are able to block adhesion, and even displace pathogens to prevent their colonization of the vaginal vault (Osset et al., 2001; Vesterlund et al., 2006).

Certain strains of *Staphylococcus aureus* produce toxic shock syndrome toxin-1 (TSST-1), which can cause menstrual toxic shock syndrome (mTSS) in women (Bergdoll et al., 1981; Schlievert et al., 1981). TSST-1 is able to cross mucosal surfaces and subsequently enter the bloodstream (Dinges et al., 2000). The toxin is a superantigen capable of activating a significant percentage of T-cells, regardless of their antigen specificity, to cause high fever, rash, and shock that can ultimately lead to death of the host (Dinge et al., 2000). Toxic shock syndrome (TSS) was reported as early as 1927, but became prominent when the number of mTSS cases increased

dramatically in the late 1970s (Shands et al., 1980). This mTSS increase was found to be associated with the use of a newly developed superabsorbent tampon by menstruating women (Shands et al., 1980). The number of cases of mTSS decreased significantly when the product was removed from the market, while the number of nonmenstrual cases has remained relatively constant (Chesney, 1997). Currently, mTSS in the United States is rare and this low incidence rate has remained somewhat constant over the past ten years (CDC, 2013). However, given the rather strict case definition of the Centers for Disease Control and Prevention (CDC), this low rate is more than likely an underestimation of the actual number of cases (CDC, 1997). For example, a study conducted in the Minneapolis-St. Paul, Minnesota area for the years 2000-2003 suggested that staphylococcal TSS was on the rise (Gaventa et al., 1989; Schlievert et al., 2004). This study utilized a broader case definition than that put forth by the CDC and determined a rise in incidence from 0.8/100,000 in 2000 to 3.4/100,000 in 2003 for women of menstrual age in the Twin Cities area (DeVries et al., 2011; Schlievert et al., 2004). This rise in incidence agreed with an 18% rise in incidence that the CDC reported for the entire United States from 2002 to 2003 (Schlievert et al., 2004). The reason for this increase in incidence is unknown, and to date has not been investigated. However, the increase may indicate an underlying reemergence of TSS caused by *S. aureus*. This is particularly alarming, since women have menstrual periods for 30 to 40 years of their life and as many as 70% of women in the United States, Canada, and much of Western Europe utilize tampons at some point during menstruation (Schlievert et al., 2004). From a microbiological standpoint, it is thought that mTSS develops from a site of colonization and not by infection. This is particularly relevant considering the fact that colonization by *S. aureus* of one or more body sites (nose, vagina, or anus) among menstruating women is as high as 50% (Parsonnet et al., 2008; Parsonnet et al., 2005). While the number of

TSST-1-producing strains found in these studies was low, the propensity for *S. aureus* to acquire the gene (*tst*) for TSST-1 is great because the gene is encoded on a highly mobile genetic element (Novick, 2003; Subedi et al., 2007). The concern is compounded by the fact that most strains of *S. aureus* encountered in the healthcare setting (hospital-acquired MRSA) are resistant to multiple antibiotics and those encountered in the community (community-associated MRSA) tend to be more virulent (David and Daum, 2010).

Many *Lactobacillus* species are capable of inhibiting *S. aureus* growth or the production of specific factors by *S. aureus* in commercially available media with or without modification (Aslim and Kilic, 2006; Dembélé et al., 1998; Elkins et al., 2008; Laughton et al., 2006; Osset et al., 2001; Otero and Nader-Macias, 2006; Vesterlund et al., 2006). Given this ability to inhibit *S. aureus*, *Lactobacillus* species have the potential to be exploited as biotherapeutic agents in the vaginal environment to prevent mTSS (Falagas et al., 2007, Marelli et al., 2004, Reid and Bruce, 2006).

The fact that the growth requirements of *Lactobacillus* species vary from strain to strain complicates the development of a medium that simulates vaginal secretions. Defined media for *Lactobacillus* species have often been created specifically for a particular species or subspecies, and even within these, some requirements are strain-dependent (Hébert et al., 2000, Hébert et al., 2004, Saguir and de Nadra, 2007, Torino et al., 2005). Development of a single medium to support sufficient growth of multiple *Lactobacillus* species found in the vaginal tract would facilitate studies examining microbiota interactions in this environment. This report adapts a chemically defined medium (Geshnizgani and Onderdonk, 1992) by adding supplements to yield adequate growth of *Lactobacillus* species and TSST-1 producing strains of *S. aureus*. This medium supports the production of TSST-1 toxin by *S. aureus* and has provided an excellent

foundation for investigating the interactions between *Lactobacillus* species and *S. aureus* isolates (Liu et al., 2011).

2. Methods

2.1. Bacterial strains, strain maintenance, and growth conditions

Strains used in this study are listed in Table 1. Clinical *S. aureus* strains isolated from cases of mTSS (P.D. Fey, University of Nebraska Medical Center) were confirmed to produce TSST-1 by western analysis. *S. aureus* isolates were maintained as frozen (−80°C) stocks in brain heart infusion (BHI; Difco Laboratories, Detroit, MI) broth containing 25% (w/v) glycerol and streaked for isolation on either tryptic soy broth (TSB; Difco Laboratories) or BHI containing 1.5% agar. *Lactobacillus* species were maintained as frozen stocks in Lactobacilli deMan, Rogosa and Sharpe (MRS) broth (Difco Laboratories) containing 10-15% (w/v) glycerol and streaked for isolation on MRS containing 1.5% agar. Strains were routinely grown overnight (15-18 h) at 37°C, with rotary aeration (180 rpm) for *S. aureus* or static anaerobic conditions for *Lactobacillus* species, in the liquid medium to be tested for each experiment. Anaerobic conditions consisted of an atmosphere of approximately 90% N₂, 5% H₂ and 5% CO₂ in an environmental chamber (Coy Laboratory Products, Inc., Grass Lake, MI) containing Pd catalyst with O₂ and H₂ analyzer.

Overnight (15-18 h) cultures were used to inoculate triplicate wells of 96-well plates (Becton Dickinson, Franklin Lakes, NJ) to an initial optical density of approximately 0.05 at 550 nm. Bacterial growth was monitored over 24 h using a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA) at 37°C, with 5-10 sec of shaking before each reading. For *S. aureus* growth, the plates were aerated by constant shaking between readings. Growth was also measured by inoculating 50 ml flasks containing 20 ml of medium to an initial optical density of

approximately 0.05 at 550 nm. Flasks were incubated statically at 37°C and at 2, 4, 6, 8, and 24 h portions were removed, diluted in the appropriate medium, and used to determine growth spectrophotometrically. Two or more biological replications for each growth determination were performed and representative growth curves are presented in the Results section.

2.2. Genital tract secretion (GTS) media

Attempts to use the original chemically-defined medium (G&O) simulating genital tract secretions developed by Geshnizgani and Onderdonk (1992) as well as G&O medium containing 0.1% glucose instead of 1.0%, to grow *S. aureus* and *Lactobacillus* species were unsuccessful (Supplemental Table).

Six other modified media were also tested (Supplemental Table). CDM+13 was CDM supplemented with amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, proline, serine, threonine, tryptophan, and valine) previously detected in vaginal secretions (Gregoire et al., 1959). CDM+21 was composed of CDM supplemented with all amino acids at 1X concentration. CDM+21+UP was composed of CDM+21 supplemented with uracil and pyruvate, and CDM+21+UPI had the same composition as CDM+21+UP but with the addition of inosine. CDM+4X21+UPI was CDM+21+UPI with a 4-fold increase in the amino acid concentrations. Modified GTS (mGTS) medium (Liu et al., 2011) was CDM+4X21+UPI with the addition of MnCl₂, sodium acetate, and an increase in glucose to 0.5%. Portions of the medium were prepared separately, as shown in Supplemental Table, and mixed aseptically, allowing the composition of the medium to be easily adjusted.

2.3. Spent medium preparation

Spent media were collected at various time points from *S. aureus* strains grown in various media and prepared essentially described by Jones et al. (2008). Briefly, at each time point, cell

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4 158 cultures were standardized by adjusting optical densities with fresh sterile media. Cells were
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7 159 removed by centrifugation and spent media were filter-sterilized using a 0.2 µm filter,
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10 160 immediately frozen using dry ice and ethanol, and stored at –80°C. Spent media were thawed on
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12 161 ice and concentrated at 6,500 x g at 4°C for 20 h using Centricon YM-3 ultracels (Millipore,
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14 162 Billerica, MA). Volumes of concentrated spent media as well as concentrated sterile media were
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17 163 adjusted to yield 10X concentrates and stored at –80°C until used.

18 19 164 *2.4. Western analysis* 20 21

22 165 In some experiments, standardized spent media were spotted as 2 µl aliquots onto
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24 166 polyvinylidene fluoride (PVDF) membranes (Amersham Hybond-P; GE Healthcare Bio-
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27 167 Sciences Corp., Piscataway, NJ) that had been pre-wetted in methanol for 2-3 sec and then
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29 168 soaked in phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH
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32 169 7.4) for 2 min. Membranes were blocked overnight with normal rabbit serum (Toxin
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34 170 Technology, Inc., Sarasota, FL) in PBS-Tween (0.05% Tween 20 in PBS). Affinity-purified,
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37 171 polyclonal anti-TSST-1 antibody conjugated to horseradish peroxidase (HRP; Toxin
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39 172 Technology) and the substrate 4-chloro-1-naphthol were used to detect TSST-1. In these
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41 173 experiments, purified TSST-1 (Toxin Technology) was serially diluted 2-fold and used as a
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44 174 positive control. In some experiments, proteins in standardized spent media were resolved using
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46 175 4-12% gradient gels (NuPAGE Bis-Tris Mini gels, Novex, Invitrogen Life Technologies,
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49 176 Carlsbad, CA) as recommended by the manufacturer (Invitrogen Life Technologies) and
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51 177 transferred to PVDF membranes (GE Healthcare Bio-Sciences) using the semidry method for
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54 178 transfer of proteins as recommended by the manufacturer (Bio-Rad, Hercules, CA). Membranes
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56 179 were blocked as described above and TSST-1 toxin was detected using the chemiluminescent
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substrate Lumingen PS-3 as recommended by the manufacturer (Amersham ECL Prime Western Blotting Detection System, GE Healthcare Bio-Sciences).

3. Results

3.1. Growth of *S. aureus* TSST-1-producing isolates and *Lactobacillus* species in media simulating genital tract secretion

Attempts to grow *S. aureus* isolates in the medium developed by Geshnizgani and Onderdonk (1992) were unsuccessful. A CDM consisting of basal medium, albumin, vitamins, additives (minus MnCl_2 and sodium acetate), and a glucose concentration of 0.1% (Supplemental Table) was developed and growth of *S. aureus* in CDM, and in MRS, was compared to growth in TSB, a medium routinely used to grow *S. aureus*. As expected, all *S. aureus* isolates (Table 1) grew well in TSB (Figures 1 and 2) with growth curves similar to that of *S. aureus* NCH 245 (MN8 1980 MN, a prototypic TSST-1-producing strain isolated from a clinical case of mTSS, Peterson et al., 2005, Schlievert and Blomster, 1983, Yarwood and Schlievert, 2000) (Figure 1A). In MRS, growth patterns varied among the *S. aureus* isolates, but all isolates grew more slowly and to a lower overall density than in TSB (Figures 1A and 2). In CDM (Supplemental Table), *S. aureus* TSST-1 isolates (Figures 1A and 2) did not grow much like the results obtained with the original medium developed by Geshnizgani and Onderdonk (1992). However, of all of the isolates examined, *S. aureus* MN8 (Figure 1A) and NCH 264 (Figure 2D) strains did exhibit some growth, albeit at a much reduced rate and an overall decreased density as compared to growth in TSB and MRS over the same period of time.

To enhance the growth of *S. aureus* TSST-1-producing isolates in CDM, amino acids that have previously been detected in vaginal secretions (Gregoire et al., 1959) (Supplemental Table), were added to CDM at concentrations for optimal production of TSST-1 (Schlievert and

Blomster, 1983) or for overall growth of *S. aureus* (Jacobsen and Smith, 1968, Wilkinson, 1997). The addition of these amino acids (CDM+13) improved the growth rate and overall density of *S. aureus* MN8 (Figure 1A). Adding taurine, to further simulate vaginal secretions, and/or phenylalanine, to promote TSST-1 production, did not appreciably affect this improvement above that observed in CDM+13 alone (Figure 1A).

While some *Lactobacillus* species grew poorly in CDM (Figure 3), most did not grow at all (data not shown). Attempts to improve *Lactobacillus* species growth were not successful in CDM+13. However, when all 21 amino acids (CDM+21) were added, growth rates and overall densities of *Lactobacillus* species improved as compared to CDM (Figure 3) and CDM+13 (data not shown) and the growth rate for *S. aureus* MN8 also improved (Figure 1B).

Uracil and pyruvate were added to CDM+21 to promote growth of *S. aureus* under anaerobic conditions (Evans, 1975; Flood, et al., 2004; Richardson, 1936) and inosine was also added to CDM+21 since published defined media for *Lactobacillus* contain nucleotides (Hébert et al., 2004; Saguir and de Nadra, 2007; Savijoki et al., 2006), a requirement that can be met by the addition of uracil and inosine (Elli et al., 2000). As expected, growth rate and overall growth yield at 24 h for *S. aureus* MN8 were less when grown in an anaerobic environment (Figure 4). However, under anaerobic conditions increases in rate and yield for *S. aureus* MN8 were observed in CDM+21 containing uracil, pyruvate, and inosine (Figure 4). Increased growth rates of some *Lactobacillus* species, for example, *L. plantarum* WCFS-1, *L. rhamnosus* 53103, and *L. gasseri* 9857, were observed in CDM+21 containing uracil, pyruvate, and inosine when compared to CDM alone (Figure 3).

The growth patterns of some *Lactobacillus* species indicated that a required factor(s) was depleted too quickly in CDM+21+UPI, resulting in low final optical densities by 24 h as

compared to growth in MRS (Figure 3). To determine which component(s) of the medium might be limiting growth, the concentrations of various components were increased and *Lactobacillus* growth was compared in the resulting media (data not shown). The best overall growth improvement of most *Lactobacillus* species, including *L. plantarum* WCFS-1, *L. rhamnosus* 53103, and *L. gasseri* 9857 (Figure 4), and of *S. aureus* TSST-1 isolates (Figures 1B and 2), was achieved when the concentrations of amino acids were increased 4-fold (CDM+4X21+UPI, Supplemental Table). The medium (CDM+4X21+UPI) was further supplemented with increasing concentrations of glucose as well as the addition of MnCl₂ and sodium acetate in an attempt to further improve growth of *Lactobacillus* species. While increasing the glucose concentration from 0.1% to 0.5% showed no appreciable increase in either growth rate or final cell density of *L. plantarum* WCFS-1, an increase in glucose concentration to either 0.25% or 0.5% with the addition of 0.005% MnCl₂ resulted in an improved growth rate as well as cell density by 24 hours (Figure 5).

The final medium composition examined in this study was mGTS medium (Liu et al., 2011) which consists of CDM+4X21+UPI supplemented with 0.5% glucose, 0.005% MnCl₂, and 0.1% sodium acetate (Supplemental Table). Growth of *L. plantarum* WCFS-1 in mGTS resulted in a growth rate comparable to that in MRS and a subtle increase in cell density by 24 h (Figure 5). This medium was then used to assess growth of a number of *Lactobacillus* species as well as *S. aureus* NCH 239 (587; Schlievert et al., 1981), 240 (FRI 1169; Reiser et al., 1983), and 245 (MN8), which are TSST-1-producing strains, and a non-TSST-1-producing strain, *S. aureus* NTH 13 (UAMS-1, a clinical osteomyelitis strain, Gillaspay et al., 1995). *Lactobacillus plantarum* WCFS-1, *L. gasseri* 9857, and *L. fermentum* 23271 exhibited growth rates comparable to those in MRS (Figures 6A and 6B). The growth rates of the remaining

Lactobacillus species examined were about the same as in MRS (Figures 6A and 6B). *Lactobacillus plantarum* WCFS-1, *L. acidophilus* 2124, *L. jensenii* 11305, *L. johnsonii* 533, *L. rhamnosus* 53103, and *L. gasseri* 19992 when grown in MRS had a consistent two-hour lag (Figures 6A and 6B). This growth characteristic was not observed in mGTS.

A select group of four *S. aureus* strains grew well in mGTS medium even though growth rates were somewhat lower in mGTS than in TSB (Figure 7). However, cell densities in mGTS by 24 were essentially the same as that achieved when grown in TSB (Figure 7).

3.2. TSST-1 production in media simulating genital tract secretion

Staphylococcus aureus MN8 (Peterson et al., 2005, Schlievert and Blomster, 1983, Yarwood and Schlievert, 2000) was grown in BHI (Schlievert and Blomster, 1983) to determine at what point in growth TSST-1 is optimally detected (Figure 8A). Spent media were collected at 3, 6, 12, and 24 h, concentrated, and analyzed for TSST-1 by dot blot western analysis. TSST-1 was detected at all time points, but its concentration was greatest at 12 and 24 h (Figure 8A).

Early in our studies, *S. aureus* MN8 was grown in BHI, MRS, CDM+21 and CDM+21+UP to assess the ability of these media to support the production of TSST-1. Spent media were collected at 24 h from each culture and concentrated 10-fold. Production of TSST-1 was detected in each medium, except MRS (Figure 8B). As expected, the highest concentration of TSST-1 was detected in BHI (Schlievert and Blomster, 1983). The addition of uracil and pyruvate to CDM+21 did not affect the production of TSST-1 at 24 h (Figure 8B).

Gradient gel electrophoresis and western analysis of standardized spent media isolated from *S. aureus* MN8 grown in a variety of media were utilized to assess protein profiles and TSST-1 levels after the CDM+21+UP medium had been extensively modified to support the growth of *Lactobacillus* species. As expected, protein profiles varied dramatically depending upon the

medium used (Figure 9A). The original Geshnizgani and Onderdonk (1992) medium (Figure 9A, lane 2) and the CDM medium developed in our laboratory (Figure 9A, lane 3) consistently exhibited protein profiles with little to no discernible protein bands except for the two prominent bands of approximately 55-66 kDa (Figure 9A, lanes 2 and 3). These bands were observed in all media examined, excluding TSB, and most likely represent the bovine serum albumin (Hirayama et al., 1990) component of the medium (Figure 9A). These media, along with CDM+, did not support the production of TSST-1 by *S. aureus* MN8 to a level that was detectable by western analysis (Figure 9B, lanes 2, 3, and 4). While the remaining media assessed, which included CDM+21, CDM+21+UPI, CDM+4X21+UPI, and mGTS, exhibited a variety of protein profiles (Figure 9A), they all supported the production of TSST-1 by the *S. aureus* MN8 strain to levels detectable by western analysis (Figure 9B). Toxin levels in TSB (Figure 9B, lane 1) were considerably higher than in any other supportive medium (Schlievert and Blomster, 1983), while the medium, mGTS, which best supported growth of *Lactobacillus* species as well as *S. aureus* TSST-1-producing strains, yielded the least amount of TSST-1 produced (Figure 9B, lane 8). Chemically-defined media containing all 21 amino acids with (CDM+21+UPI) or without (CDM+21) uracil, pyruvate, and inosine supported TSST-1 production to levels approaching that observed in TSB (Figure 9B, lanes 5 and 6). Both these media contain 0.1% glucose and we suspected the increase in glucose concentration in mGTS to 0.5% most likely resulted in the observed reduction in TSST-1 production (Figure 9B, lane 8) (Schlievert and Blomster, 1983). The reduced expression of TSST-1 by the *S. aureus* MN8 strain in CDM+4X21+UPI was somewhat surprising since this medium contains 0.1% glucose (Figure 9B, lane 7). However, CDM+4X21+UPI does contain an increase in amino acid concentration to 4-fold thus, this increase in concentration may account for the reduced levels of TSST-1 (Figure 9B, lane 7).

To determine whether this decrease in TSST-1 in CDM+4X21+UPI was strain-dependent, proteins from standardized spent media isolated from a select group of TSST-1-producing strains of *S. aureus* grown in CDM+4X21+UPI were analyzed for TSST-1 production (Figure 10). Both protein profiles and TSST-1 levels were strain-dependent as indicated by a variety of protein profiles and low TSST-1 production by NCH 242 to high TSST-1 production by NCH 240 (FRI 1169).

4. Discussion

The current study assessed a previously reported simulated genital tract secretion medium (Geshnizgani and Onderdonk, 1992) to support dense growth of eight *Lactobacillus* species and 27 strains of *S. aureus*, including the prototypic TSST-1-producing strain, MN8. This medium, a chemically-defined medium, uses albumin as the sole source of most amino acids, although cysteine was used as a reducing agent (Geshnizgani and Onderdonk, 1992). Attempts to grow *S. aureus* and several species of *Lactobacillus* in this medium along with our initial adaptation of this medium were unsuccessful, at least to densities that would represent high cell number. To determine whether free amino acids were required for abundant growth, the 13 amino acids that have been identified in human vaginal fluid were initially added. Although taurine is not used in protein production, it was detected in vaginal secretions (Gregoire et al., 1959), so it was included in the media. Phenylalanine has not been reported in vaginal secretions, but was included for optimal TSST-1 production (Schlievert and Blomster, 1983). Although the addition of limited amino acids somewhat improved growth of *S. aureus* TSST-1 isolates, growth of *Lactobacillus* species was not improved. Since defined media for *Lactobacillus* species include all 20 amino acids (Elli et al., 2000; Savijoki et al., 2006), the remaining amino acids were added, which significantly improved the growth of all bacteria examined. Further modifications

of the medium to include the addition of uracil, pyruvate, inosine, MnCl_2 , and sodium acetate along with an increase in glucose concentration to near physiological levels (Sumawong et al., 1962) supported the growth of *S. aureus* isolates almost as well as TSB, and greatly improved the growth of a number of *Lactobacillus* species to levels comparable to growth observed in MRS. While this study did not determine viable cell counts, the optical densities achieved in media, such as CDM+4X21+UPI and mGTS, most likely represents the relatively high cell numbers that can be detected in the human vaginal tract (Bartlett et al., 1977; Chase et al., 2007 and 2010; Eschenbach et al., 1989; Onderdonk et al., 1986 and 1987). In these studies, a variety of vaginal sampling techniques were used with human volunteers at various stages of the menstrual cycle to consistently demonstrate *Lactobacillus* species concentrations ranging from $10^7 - 10^9$ cfu/ml. In addition, these studies demonstrated that when present, *S. aureus* numbers could be as high as 10^6 cfu/ml without clinical presentation of mTSS.

Toxin production by *S. aureus* in these media varied both with media composition and strain examined. Toxin was only detected by western analysis when the CDM contained all 21 amino acids, a result most likely attributed to the inclusion of phenylalanine (Schlievert and Blomster, 1983). While mGTS may have contained a composition optimal for both *Lactobacillus* species and *S. aureus* strain growth, TSST-1 production, though detectable by western analysis in this medium, was greatly reduced as compared to levels produced in TSB and in other modified versions of CDM that did not contain MnCl_2 and sodium acetate or an increase in glucose concentration but did contain all amino acids.

We have developed a defined medium (mGTS) that simulates vaginal secretions and supports the growth of several *Lactobacillus* species and of *S. aureus* TSST-1-producing isolates as well as the production of TSST-1. This medium will provide an excellent environment for further

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4 341 studies to examine the effects of *Lactobacillus* species on *S. aureus* growth and TSST-1
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Table 1.

Bacterial strains.

Strain	Relevant characteristic	Source
<i>S. aureus</i>		
NCH 239 (587 CDC 1980), 240 (FRI 1169 1980 WI), 241 – 244, 245 (MN8 1980 MN), and 246 – 264	Vaginal TSS	P.D. Fey, UNMC ¹
NTH 13 (UAMS-1)	Osteomyelitis	M.S. Smeltzer, UAMS ²
<i>L. acidophilus</i> JCM 2124	Human vagina	JCM ³
<i>L. delbrueckii</i> ATCC 11842	Yogurt	ATCC ⁴
<i>L. fermentum</i> ATCC 23271	Human intestine	ATCC
<i>L. gasseri</i> ATCC 9857	Human vagina	ATCC
<i>L. gasseri</i> ATCC 19992	Human feces	ATCC
<i>L. jensenii</i> JCM 11035	Vagina	JCM
<i>L. johnsonii</i> NCC 533	Probiotic	NCC ⁵
<i>L. plantarum</i> WCFS-1	Human saliva	WCFS ⁶
<i>L. rhamnosus</i> ATCC 53103	Human feces	ATCC

¹University of Nebraska Medical Center, Omaha, NE

²University of Arkansas for Medical Sciences, Little Rock, AR

³Japanese Collection of Microorganisms, Tsukuba, Japan

⁴American Type Culture Collection, Manassas, VA

⁵Nestlé Culture Collection, Lausanne, Switzerland

⁶Wageningen Center for Food Sciences, Wageningen, Netherlands

FIGURE LEGENDS

Figure 1: Representative growth curves of *S. aureus* strain NCH 245 (MN8). (A) Growth in TSB, MRS, CDM, CDM+13, CDM+13+Taurine (Tau), CDM+13+Phenylalanine (Phe), and CDM+13+Tau+Phe media. (B) Growth in CDM+21, CDM+21+uracil (U)+pyruvate (P), CDM+21+UP+inosine (I), and CDM+4X21+UPI media.

Figure 2: Representative growth curves of *S. aureus* strains NCH 240 (FRI 1169) (A), 242 (B), 260 (C), and 264 (D) in TSB, MRS, CDM, and CDM+4X21+UPI.

Figure 3: Representative growth curves of *S. aureus* strain NCH 245 (MN8) in TSB, CDM+21, CDM+4X21+UPI, and mGTS media under aerobic and anaerobic conditions.

Figure 4: Representative growth curves of *L. plantarum* WCFS-1 (A) and *L. rhamnosus* 53103 (B), and *L. gasseri* 9857 (C) strains in MRS, CDM, CDM+21, CDM+21+UP, CDM+21+UPI, and CDM+4X21+UPI media.

Figure 5: Representative growth curves of the *L. plantarum* WCFS-1 strain in MRS, CDM+4X21+UPI containing either glucose or glucose and MnCl₂, and mGTS media.

Figure 6: Representative growth curves of *Lactobacillus* species in MRS and mGTS media. (A) *L. plantarum* WCFS-1, *L. gasseri* 9857, *L. acidophilus* 2124, and *L. jensenii* 11305. (B) *L. johnsonii* 533, *L. rhamnosus* 53103, *L. fermentum* 23271, and *L. gasseri* 19992.

Figure 7: Representative growth curves of *S. aureus* NCH 245 (MN8), 240 (FRI 1169), 239 (587), and NTH 13 (UAMS-1) strains in TSB and mGTS media.

Figure 8: Production of TSST-1 by the *S. aureus* NCH 245 (MN8) strain. Spent media and purified toxin were spotted onto PVDF membranes and hybridized with anti-TSST-1. (A) Concentrated BHI spent media isolated at 3, 6, 12, and 24 hr of growth. (B) A two-fold serial dilution of either purified TSST-1 toxin or 10X concentrated spent media isolated from the *S.*

aureus NCH 245 (MN8) strain at 24 hr. Purified TSST-1 toxin (1), sterile CDM+21 media (2), BHI spent media (3), MRS spent media (4), CDM+21 spent media (5), CDM+21+UP spent media (6), and sterile CDM+21+UP (7).

Figure 9: Extracellular protein profiles (A) and TSST-1 western analysis (B) of spent media isolated at 24 hr from the *S. aureus* NCH 245 (MN8) strain grown aerobically in various formulated media. Media were TSB (1), CDM (Geshnizgani and Onderdonk, 1992) (2), CDM (3), CDM+13 (4), CDM+21 (5), CDM+21+UPI (6), CDM+4X21+UPI (7), and mGTS (8). M, molecular mass marker in kDa (A) and asterisk denotes purified TSST-1 protein (B). Arrows indicate a tentative identification of bovine serum albumin. Arrowhead indicates the relative position of TSST-1 (22 KDa, Schlievert et al., 1981).

Figure 10: Extracellular protein profiles (A) and TSST-1 western analysis (B) of spent media isolated at 24 hr from *S. aureus* NCH 239 (587 CDC 1980) (1), 240 (FRI 1169) (2), 241 (3), 242 (4), 254 (5), 260 (6), 261 (7), and 263 (8) strains grown aerobically in CDM+4X21+UPI. M, molecular mass marker in kDa (A) and asterisk denotes purified TSST-1 protein (B). Arrows indicate a tentative identification of bovine serum albumin. Arrowhead indicates the relative position of TSST-1 (22 KDa, Schlievert et al., 1981).

Figure 1A.

Figure 1B.

Figure 2A.

Figure 2B.

Figure 2C.

Figure 2D.

Figure 3A.

Figure 3B.

Figure 3C.

Figure 4.

Figure 5.

Figure 6A.

Figure 6B.

Figure 7.

Figure 8A.

Figure 8B.

Figure 9.

Figure 10.