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Characterization of microbial contaminants in urine

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Modern, molecular microbiological methods were applied to urine samples from control subjects and athletes for characterization of the microbial community. High abundance of lactobacilli, enterococci, and enterobacteria was detected in urine samples, suggesting that gastrointestinal and urogenital tracts act as contamination sources. Athlete samples, but not control samples, showed an abundance of pseudomonads, a bacterial group reported to metabolize steroids. Overall, the bacteria detected are known to be capable of altering steroid profiles, emphasizing the importance of good hygiene at sampling in reliable doping control.

Introduction

In sports doping control, urine analysis to detect abuse of 'natural' androgenic steroids, such as testosterone, relies largely on alterations in the endogenous steroid profile. The capability of micro-organisms to alter steroid profiles has long been recognized,[1] but only during the past decade have the effects of microbiological contamination and the stabilization of urine samples been studied.[2–8]

In the bladder, urine is sterile, but it can be contaminated while leaving the urinary tract and when exposed to external microbial load. As a result, enzyme activity generated by micro-organisms may cause changes in the natural androgenic steroid profiles by increasing or decreasing the concentrations of various steroids or by hydrolyzing conjugated metabolites.[1] Steroid profiles can be altered due the formation of 5α-androstanedione and 5β-androstanedione from androsterone and etiocholanolone glucuronides via bacterial transformation. Bacterial metabolism has also been reported to increase testosterone concentrations, leading to elevated testosterone to epitestosterone (T/E) ratios.[2] Apart from changes in endogenous steroid profiles, micro-organisms have been reported to produce low levels of nandrolone metabolites from endogenous androsterone and etiocholanolone.[8] Endogenous production of boldenone has been shown to be a consequence of bacterial metabolism, but disappearance of exogenous boldenone has also been observed.[9–11] The risk of false-negative or even false-positive results due to the microbial transformation of chemical substances in urine must be excluded in sports doping control.

Several studies have been carried out on the stabilization of human urine samples.[12–14] None of the physiological methods investigated, including sterilization by filtration, ultraviolet radiation, or ultrasonication, have succeeded in preventing microbial growth. Chemical methods have been shown to be more efficient, but the introduction of any chemical substance into athlete samples after collection may be difficult to justify legally. Consequently, rapid freezing has proved to be the only feasible method for stabilizing samples and preventing microbial activity. However, this procedure is not always possible during sample transportation[15] and would significantly increase the total costs of routine doping control.

Presence of microbes from, for example, the gastrointestinal or urinary tract in urine samples can result in the growth of various microbial communities, which can be assumed to be affected by the prevailing conditions in urine. External conditions, especially the temperature during storage, also have an effect on bacterial growth and the composition of bacterial communities. The influence of endogenous conditions in urine was studied back in the 1930s, when staphylococci and intestinal bacilli were inoculated together with various urine components in distilled water.[16] In the 1960s, *Escherichia coli* was shown to grow more efficiently at pH $>$ 6 than at lower pH values.[17] However, the microbial techniques of the past were mainly based on culturing, and therefore the focus was on those microbes that could be cultivated in laboratory conditions. Traditionally, suspicions of microbial growth are based on sensory observations, assessing turbidity, presence of precipitate and smell, and measurement of pH when sample bottles are opened in a doping control laboratory, but these methods are inaccurate.[18]

Modern molecular biology methods have shown that traditional microbiological methods are inefficient, because the majority of microbes cannot be cultivated under artificial laboratory conditions. Nowadays, techniques based on polymerase chain reaction and analysis of the 16Sr RNA gene provide a rapid, accurate alternative to phenotypical methods of bacterial identification. However, the molecular biology approach has not been exploited in the context of sports doping control. In a novel approach, the present study determined urine microbial levels by quantitative PCR and the composition of the microbial communities by 16S rRNA gene sequencing for a control group and a group of athletes. The technique reported here allows rapid identification

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of microbes and helps evaluate the probability of alteration of the steroid profiles in urine samples by the contaminant microbes.

Materials and Methods

A total of 94 urine samples from routine laboratory specimens was collected for microbiological characterization. The inclusion criteria were sensory observations for high turbidity, or presence of precipitate or abnormal smell (+/− scale) or elevated pH (>8). The samples included 60 urine samples collected from athletes in routine doping control (hereafter referred to as 'athlete samples') and 34 control samples collected for drug screening (hereafter referred to as 'control samples').

Bacterial chromosomal DNA was extracted from the samples and total microbial levels were analyzed with quantitative real-time polymerase chain reaction (qPCR) using the universal broad-range primer pair 536f and 907r.[19] In qPCR, the signal increases in direct proportion to the amount of amplified PCR product in the reaction. The higher the starting copy number of the nucleic acid target, the sooner an increase in fluorescence signal exceeding the threshold value is observed. Hence, the detection system allows the exponential phase to be observed in order to calculate the amount of target template present at the start, using a log-linear relationship between the standards and the threshold cycles. The structure of the microbial community in samples with elevated microbial levels was assessed with partial 16S rRNA gene sequencing. The broad-range primers 536f-907r target the conserved regions of the 16S rRNA gene in order to unselectively amplify all bacterial DNA present in the sample. The resulting sequences cover a variable region of the gene used for identification of the clone. The sequences obtained from the selected athlete samples (a total of 17 samples: 10 female and 7 male) and control samples (a total of 18 samples: 11 female and 7 male) were identified by comparing them against the RDP II sequence database.[20]

Results and Discussion

Validity of sensory observations and pH as markers to predict microbial levels

Sensory observations and pH did not appear to be useful markers to predict microbial levels, as the parameters high turbidity, presence of precipitate, abnormal smell, and pH were not correlated to measured microbial levels. Samples with measurable, but low, microbial levels (from 5×10^5 to 1×10^8 16S copies/ml) and samples with high microbial levels ($>1 \times 10^8$ 16S copies/ml) did not differ in terms of sensory observations. The pH was higher in control samples with high microbial levels than in control samples with measurable, but low, microbial levels ($p < 0.01$). However, in athlete samples and the combined dataset, no difference in pH was observed as regards microbial level. The subjective nature of sensory observations weakens their validity as markers for microbial contaminants, while the poor validity of pH is understandable when the effect of fermentation type on pH is considered. If microbes are present in the urine sample, the pH may either decrease due to microbial production of acids (short chain fatty acids) or increase due to microbial production of basic compounds (ammonium and amines).[21]

Microbial levels in urine samples

The microbial levels in urine samples ranged from below the detection limit ($<5 \times 10^5$ 16S copies/ml) to as high as 5×10^9 16S copies/ml. The 16S copy number/microbial cell varies between different microbes but assuming an average value of 5,[22] the highest levels represent $\sim 1 \times 10^9$ cells/ml, which corresponds to the stationary phase (highest practically possible) of liquid cultures. It is worth noting that transport of samples to doping laboratories may take days at ambient temperature, and therefore low initial microbial contamination can result in a high final microbial level. Assuming a generation time of 60 min (which clearly exceeds the shortest generation times of 20 min reported for certain enterobacteria), initial contamination by 100 bacterial cells would reach a theoretical level of 10^9 cells/ml within 24 h. Of the 34 control samples, 15 samples (44%) showed values below the detection limit, 13 samples (38%) had levels between 5×10^5 16S copies/ml and 1×10^8 16S copies/ml and 6 samples (18%) had levels exceeding 1×10^8 16S copies/ml. The corresponding figures for the athlete samples were 34/60 (56%), 16/60 (26%) and 10/60 (20%), respectively, indicating a similar distribution in control and athlete samples.

The incidence of samples with measurable microbial levels was higher in females than in males. For the athlete samples, 68% (13/19) of the female samples and only 32% (13/41) of the male samples showed microbial numbers above the detection limit ($p < 0.01$ with Wilcoxon rank-sum test). In control samples, this phenomenon was even more obvious: 92% (12/13) of the female samples and only 36% (8/22) of the male samples showed microbial levels above the detection limit. This observation is in line with the fact that females are more susceptible to urinary infections.[23] The differences in microbial levels between the sexes are also linked to the composition of the microbial community in the samples.[24]

Characterization of microbial communities in urine samples

The samples with the highest microbial levels as measured by qPCR were sequenced for the partial 16S gene. A total of 35 sequence libraries was constructed, 17 from athlete samples and 18 from control samples. The number of sequences within the libraries varied between 43 and 48. Table 1 lists microbes comprising at least 10% of total bacteria in one or more samples.

The vast majority of sequences in both control and athlete samples belonged to well-characterized genera within the phyla *Firmicutes* and *Proteobacteria* (Table 1). Within the *Firmicutes*, species of the genus *Lactobacillus* were found in 8 control samples and 5 athlete samples. *Lactobacillus* species were detected more often in female samples than in male samples in both sample groups (the proportion of lactobacilli exceeded 10% of total bacteria in urine from 6/11 females in the control group and 5/10 females in the athlete group, while the corresponding figures for males were only 2/7 and 0/7). *Lactobacillus iners* is part of the normal vaginal flora of women,[24] but due to its complicated and partially still unknown growth requirements, it has not been acknowledged until recently.[25] In addition to *Lactobacillus iners*, the sequence libraries contained notable numbers of *Lactobacillus crispatus* which is a typical representative of both vaginal[24] and gastrointestinal flora.[26–28] These two *Lactobacillus* species in fact represented a major cluster in urine samples, as the proportion of *Lactobacillus* spp. was over 40% of total bacteria in 12 samples, over 80% in 8 samples and a full 100% in 5 samples (note that the order *Lactobacillales* presented in white in Figures 1A and 1B also contains other members in addition to the genus *Lactobacillus*).

Table 1. Microbes comprising more than 10% of total bacteria in one or more of the 35 sequence libraries. Samples denoted 'C' belong to the control group and those denoted 'A' to the athlete group. Taxonomic levels from phylum to genus are shown for all microbes, while species level identification is shown for sequences with S_{ab} score >0.95 against the RDP II sequence database. S_{ab} score is a similarity index calculated according to the algorithm in the RDP II sequence database[20]

Phylum	Class	Order	Family	Genus (and species)	Found in samples:
Actinobacteria	*Actinobacteria*	*Bifidobacteriales*	*Bifidobacteriaceae*	*Gardnerella* (*G. vaginalis*)	Females C11,A01,A07, Male C06
Bacteroidetes	*Bacteroidetes*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	Female C07, Male C01
Firmicutes	*Clostridia*	*Clostridiales*	*Incertae Sedis XI*	*Peptoniphilus*	Female C07
Firmicutes	*Clostridia*	*Clostridiales*	*Incertae Sedis XI*	Clostridial cluster XIII	Male C01
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Dialister*	Female C07
Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*	*Sporosarcina*	Male A06
Firmicutes	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Jeotgalicoccus* (*J. psychrophilus*)	Females A06,A07, Male A06
Firmicutes	*Bacilli*	*Lactobacillales*	*Aerococcaceae*	*Aerococcus*	Female A07
Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium* (*C. viridans*)	Female C06
Firmicutes	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus* (*E. dispar*)	Female C10, Males C04,C07,A04
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus* (*L. iners*)	Females C04,C05,C08,C09,C11,A01, A03,A07, Males C03,C06
				(*L. crispatus*)	Females C02,A05,A06
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Campylobacter* (*B. ureolyticus*)	Female C7
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia* (*E.coli*)	Females C01,C03,C06,C08,A02,A04, A09, Male C02
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella/Morganella/ Serratia*	Female A03, Males C05,A02,A03
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	Male A6
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas* (*P.fulva*)	Male A2, Females C06,A08,A10, Males A01,A07
				(*P.psychrophila/syringae*)	Females A08,A10, Male A07
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Stenotrophomonas* (*S.rhizophila*)	Males A05,A06

Lactobacilli show β-glucuronidase activity[29] and are also able to metabolize bile acids and cholesterol to produce androst-4-en-3,17-dione and testosterone.[30–32]

Within the *Proteobacteria*, different representatives of the family *Enterobacteriaceae* were present in high proportions in 6 control samples and 6 athlete samples (Table 1; Figures 2A and 2B). Similarly to *Lactobacillus iners, Escherichia coli* was more abundant in female samples than in male (comprising more than 10% of total bacteria in 4/11 and 3/10 female samples in control and athlete groups, respectively, and in only 1/7 and 0/7 male samples). *Escherichia coli* are a common cause of urinary tract infections, but also belong to the normal intestinal flora.[33] Similarly to lactobacilli, enterobacteria were always present in high proportions when detected (more than 80% of all bacteria in 10 samples and a full 100% in 7 samples; Figures 1A and 1B). The commonly observed β-glucuronidase activity within *E. coli* strains[34] may help enterobacteria to survive and grow in urine with little carbohydrates. In fact, β-glucuronidase activity has been suggested to indicate high numbers of enterobacteriaceae in doping samples.[35–36] However, the wide usage of this enzyme for hydrolysis in doping laboratories challenges its validity as a microbial marker.

Species in the genera *Prevotella* and *Enterococcus* are other common members of the gastrointestinal tract microbial community,[26–28] while the genera *Gardnerella, Peptoniphilus, Dialister, Jeotgalicoccus, Aerococcus* and *Campylobacter* contain species isolated from the urogenital tract, belonging to either the normal urogenital flora or associated with urinary infections.[37–41]

Consequently, microbial contamination from the urogenital and gastrointestinal tract during sampling can explain the presence of many of the bacteria detected (Table 1). This finding again emphasizes the importance of high hygiene levels for the samples, as a low initial microbial contamination can grow during transport to high levels of contaminating microbes, from 100 cells to

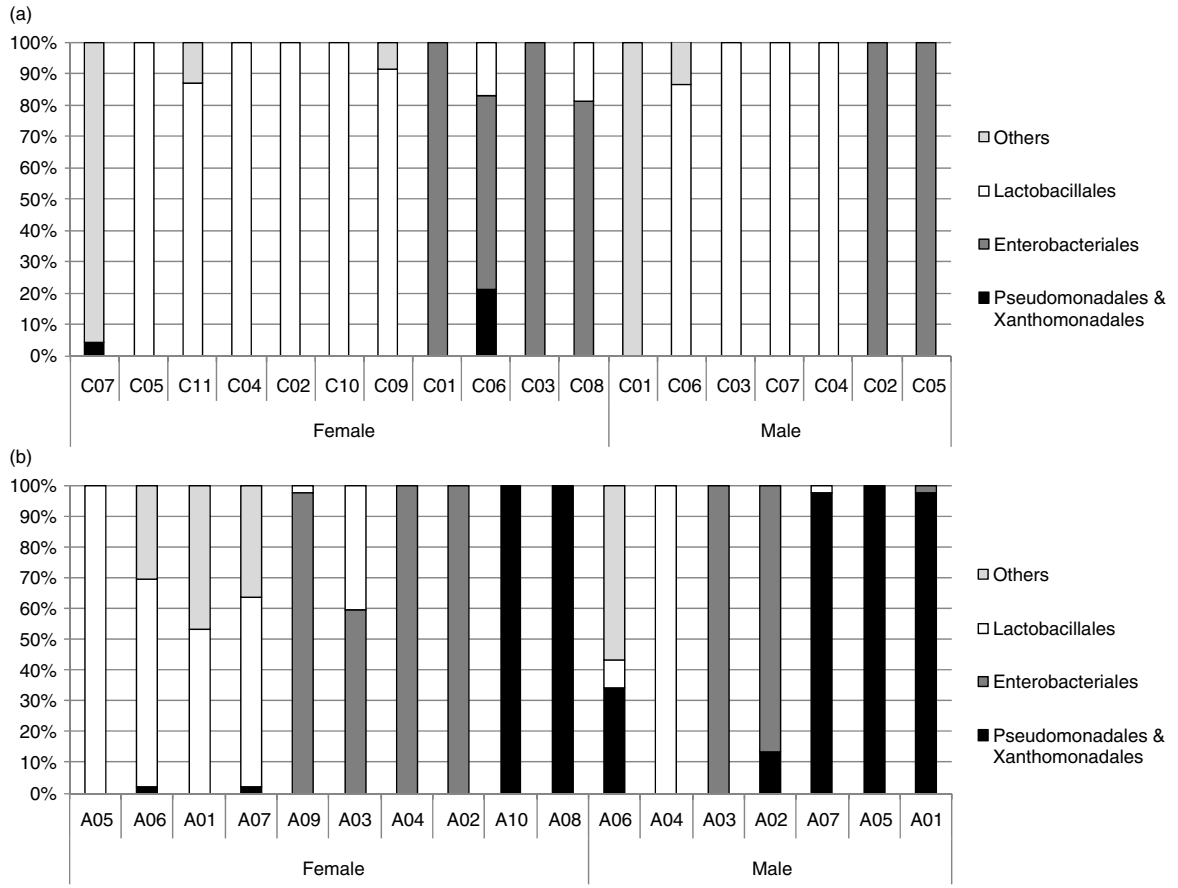

Figure 1. Percentage abundance of major clusters in a) control and b) athlete samples. Proportions of bacteria within the orders *Pseudomonadales* and *Xanthomonadales* (pseudomonads) are shown in black, within the *Enterobacteriales* (enterobacteria) in dark grey, within the *Lactobacillales* (lactobacilli) in white and within all other orders in light grey. In Figs 1 and 2, the samples are organized according to the dominant group in increasing total microbial levels, starting from samples dominated by organisms other than lactobacilli, enterobacteria and pseudomonads, followed by samples dominated by lactobacilli, enterobacteria and pseudomonads.

stationary phase within 24 h. The literature provides evidence of the capability of urogenital and gastrointestinal tract microbes to modify steroids.[29–32,34] Furthermore, it should be borne in mind that microbes prevailing in complex environments, such as the gastrointestinal tract, possess a broad range of enzymatic mechanisms that have not yet been fully identified.

Notable exceptions among bacteria from the urogenital/gastrointestinal tract are representatives of the genera *Acinetobacter, Pseudomonas* and *Stenotrophomonas* within the orders *Pseudomonadales* and *Xanthomonadales*, respectively (Table 1). For simplicity, these are referred to as 'pseudomonads' in the following text. Pseudomonads are commonly found in environmental samples, for example, water or soil samples, but can also act as opportunistic human pathogens, capable of infecting the urinary tract among others.[42–44] Species belonging to the genus *Pseudomonas* comprised more than 10% of the bacteria found in the urine samples of 4 male athletes and 2 female athletes and only one sample in the control group. In the 2 female athlete samples and 3 of the 4 male athlete samples, they comprised more than 95% of the bacterial population, indicating that these samples were practically a pure culture of *Pseudomonas* species (Figure 1B). Pseudomonads were present in significantly higher proportions in athlete samples than in control samples ($p < 0.0001$ for comparison of the two 16S rRNA gene libraries with the commercial programs Library Compare[45] and LIBSHUFF[46]). The high abundance of pseudomonads in athlete samples is of paramount importance, as pseudomonads are known to be able to metabolize steroids by various mechanisms.[1,47–49] The growth of pseudomonads in urine samples may be related to the capability of *Pseudomonas* species to utilize testosterone as the sole carbon source.[47] Pseudomonads are also known to grow using dehydroepiandrosterone as a carbon source, producing androstenedione and testosterone.[49]

Connection between microbial levels and composition of the microbial community

Figure 2 shows the total microbial levels in the samples characterized in terms of microbial community, with the dominant cluster in the sample indicated by a different colour in the column. Five female and 4 male samples in the control group and 4 female and 1 male samples in the athlete group were dominated by lactobacilli. The samples dominated by lactobacilli showed lower microbial levels than the samples dominated by enterobacteria ($p < 0.05$ for control group, athlete group and the combined data set). Females had more samples dominated by enterobacteria than males, which relates to the higher microbial levels observed for females than males. The pH was also higher in samples dominated by enterobacteria than in samples dominated by lactobacilli in the control group ($p = 0.02$), supporting the hypothesis that higher

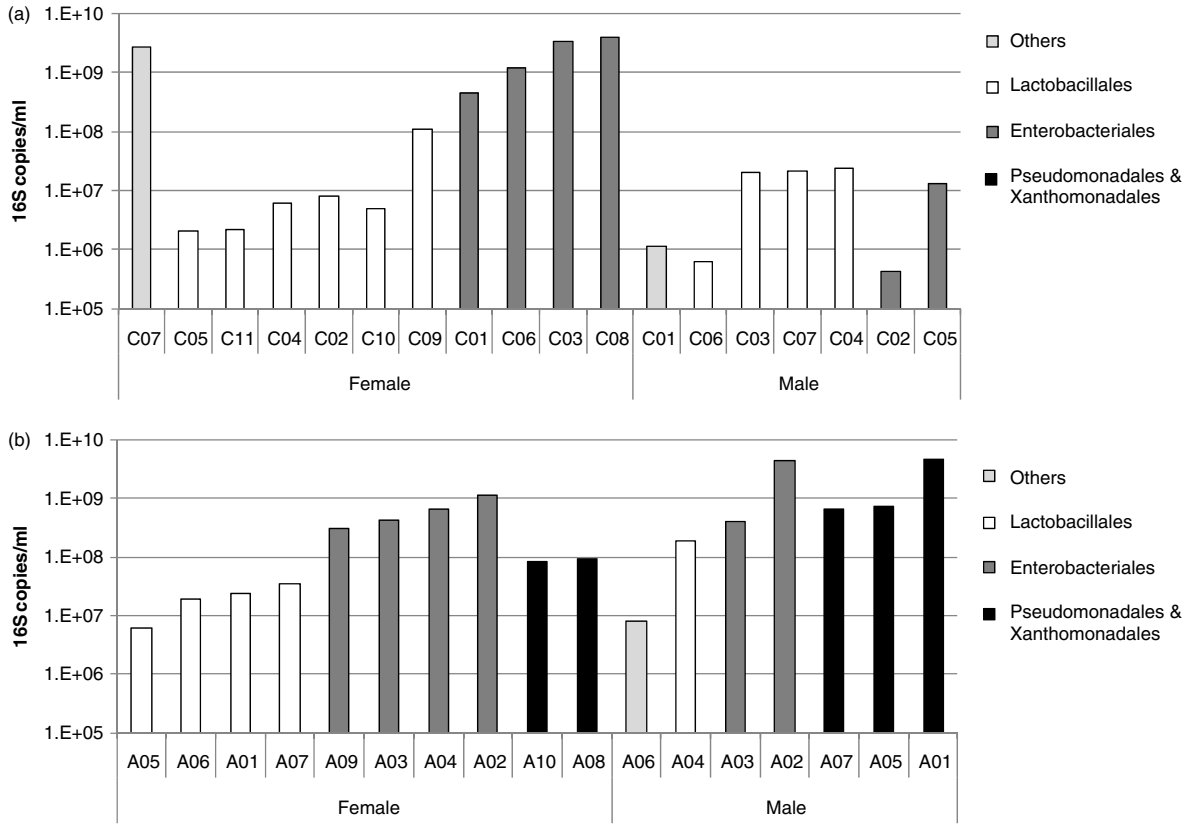

Figure 2. Total microbial levels in a) control and b) athlete samples. The colour of the column indicates the dominant cluster with the following coding: samples dominated by pseudomonads are shown in black, those dominated by enterobacteria in dark grey, by lactobacilli in white and by other organisms in light grey. Both in Figs 1 and 2, the samples are organized according to the dominant group in increasing total microbial levels, starting from samples dominated by organisms other than lactobacilli, enterobacteria and pseudomonads, followed by samples dominated by lactobacilli, enterobacteria and pseudomonads.

pH favours enterobacterial growth.[17] Similarly, the samples dominated by pseudomonads had higher microbial numbers than samples dominated by lactobacilli ($p = 0.002$ for the combined data set). These results indicate that urine provides a more suitable growth substrate for enterobacteria and pseudomonads than for lactobacilli. Amino acid synthesis is crucial for the growth of enterobacteria in urine,[50] where *E. coli* strains rapidly achieve high levels.[17,51] The capability of enterobacteria and pseudomonas to grow in urine is probably related to their deconjugation and hydrolysis activity for the endogenous human steroids.[1,34,47,49]

Conclusions

Pseudomonads were present in significant proportions in athlete urine samples, whereas they were not detected in control urine samples. This is an important finding, as *Pseudomonas* species can metabolize steroids, leading to altered steroid profiles.[1,47–49] Bacteria detected in both athlete and control urine samples were members of the families *Lactobacillaceae, Enterococcaceae* and *Enterobacteriaceae,* suggesting that the contaminating bacteria originated from the gastrointestinal and urogenital tracts. These bacteria also have the potential to modify steroid profiles.[29–32,34] A high hygiene level at sampling and maintenance of the chill chain are required, as during sample transport low levels of initial contamination may grow to reach high microbial numbers capable of modifying the steroid profiles in the samples. The known capability of microbes to alter steroid profiles[1–2,8,9–11,29–32,34,47–49] suggests that amounts of free steroids, which are already monitored by advanced doping laboratories nowadays, correlate better to microbial levels than the traditional sensory observations or pH measurement. The latter were shown to be poor indicators for bacterial growth in urine samples. Furthermore, our results question the validity of doping control in cases where high levels of microbes contaminate the urine samples, either unintentionally as a result of compromised hygiene at sampling or due to deliberate amendment by microbes to falsify the doping control results.

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