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their antibiotic-resistance profiles and their growth rate [11]. Walk et al. [12] demonstrated that the majority of the E. coli strains that are able to persist in the environment belong to the B1 phylogenetic group. Furthermore, genome size differs among these phylo-groups, with A and B1 strains having smaller genomes than B2 or D strains [13]. Johnson et al. [14] found that strains from phylo-groups B2 and D contained more virulence factors than strains from the phylo-groups A and B1.

The extraintestinal pathogenic strains usually belong to groups B2 and D [15,16], the commensal strains to groups A and B1 [17], whilst the intestinal pathogenic strains belong to groups A, B1 and D [18]. Clermont et al. [19] have developed a PCR based method to characterize the strains [13]. Johnson and B1 strains having smaller genomes than B2 or D genome size differs among these phylo-groups, with A note that strains from group B1 were found among all the phylogenetic groups among mammals is dependent on the host diet, observed that the relative abundance of phylogenetic markers chua, yjaA and the DNA fragment TspE4.C2. To increase the discrimination power of E. coli population analyses, it has been proposed the use of subgroups A0, A1, B1, B2, B23, D1 and D2, that are determined by the combination of the genetic markers [5].

Some authors analyzed the distribution of the main phylogenetic groups among E. coli strains isolated from human and animal feces. Gordon and Cowling [10] observed that the relative abundance of phylogenetic groups among mammals is dependent on the host diet, body mass and climate. Escobar-Parano et al. [5] analyzing fecal strains isolated from birds, non-human mammals and humans, observed the prevalence of groups D and B1 in birds, A and B1 in non-human mammals, and A and B2 in humans. These authors concluded that one of the main forces that shapes the genetic structure of E. coli populations among the hosts is domestication. Baldy-Chudzik et al. [20] analyzed feces from zoo animals and found a prevalence of group B1 in herbivorous animals and a prevalence of group A in carnivorous and omnivorous animals.

The aim of this work was to analyze the distribution of phylogenetic groups and subgroups in feces from different animals and to assess the potential application of this analysis in identifying the major source of fecal contamination in the environment.

Results

In this work, 241 E. coli strains isolated from feces of different animals and 12 strains isolated from a sewage source were allocated into four phylogenetic groups (i.e. A, B1, B2 and D) and seven subgroups (i.e. A0, A1, B1, B2, B23, D1 and D2). As shown in Table 1, the strains analyzed were distributed among the seven subgroups, and the prevalence indexes calculated for the subgroups were: A0 = 83.33%, A1 = 83.33%, B1 = 100%, B2 = 50%, B23 = 16.67%, D1 = 66.67% and D2 = 66.67%. It is interesting to note that strains from group B1 were found among all the analyzed hosts, whereas strains from subgroup B23 were found only in humans.

The graphic representation shown in Figure 1 allowed the identification of remarkable trends among the E. coli strains from the different hosts. Humans are the only host bearing strains from all the phylo-groups, except for subgroup A0. The strains found in the pig samples were also distributed among all phylo-groups, except for subgroup B2, which contains only strains from the human samples. Most of the strains from the chicken samples were included in subgroup A0, that is, these strains did not reveal the presence of the genetic markers investigated. Most of the strains of cows, goats and sheep fell within group B1, despite the fact that four strains of cows and three of chickens were assigned to subgroup D1 and two strains of goats and two of cows were assigned to group A1.

A Chi-square value of 97.611, 15 degrees of freedom (D.F.), p < 0.0001, was obtained from a contingency table with the phylogenetic groups distribution among the hosts, allowing the null hypothesis, which states that there is no association between the hosts and the groups, to be rejected (p < 0.0001). This result suggests a significant difference in the E. coli population structure among the animals analyzed. A Chi-square test at the subgroup level was performed to verify the existence of an association between the hosts and the phylogenetic subgroup. The calculated 155.251 Chi-square value (30 D.F.), leads to the rejection of the null hypothesis (p < 0.0001). A Chi-square test was also performed to verify the association between the hosts and the genetic markers (chua, yjaA and TspE4.C2). The result (Chi-square value = 87.563, 10 D.F., p < 0.0001) indicated that the genetic markers are differently distributed among the hosts (Table 2).

The Shannon and Simpson diversity indexes [21,22] were used to analyze the phylogenetic subgroup data. As shown in Table 3, the largest diversity indexes were observed for humans (Shannon index = 0.6598, Simpson index = 0.7331) and pigs (Shannon index = 0.6523, Simpson index = 0.7245), whilst the smallest diversity was observed for goats (Shannon index = 0.2614, Simpson index = 0.3203). The Pianka’s similarity index was calculated using the phylogenetic subgroup distribution for each pair of hosts (Table 4). The results indicated that humans and pigs exhibited a similarity of 88.3%, whereas cows, goats and sheep exhibited an average similarity of 96%.

A Correspondence Analysis (CA) was performed using the phylogenetic groups and subgroups distribution and the genetic markers distribution (Tables 1 and 2). The bidimensional representation of subgroups distribution in each host is shown in Figure 2. This bidimensional representation can explain 93.74% of the total inertia. The
horizontal axis represents 73.85% of the total inertia, which is responsible for the major separation. According to this analysis, the subgroup distribution was similar for cows, goats and sheep and for pigs and humans (Figure 2). A sewage sample was included in the CA (Figure 2). This sample included the following subgroups: A0 (one strain), A1 (five strains), D1 (four strains) and D2 (two strains). As expected, this subgroup distribution was similar to the one found for humans (Figure 2).

The CA using the genetic markers distribution resulted in a bidimensional representation that can explain 100% of the total inertia (Figure 3), being the horizontal axis responsible for 92.04% of it. According to this analysis, the genetic markers distribution was similar for cows, goats and sheep and for humans, chickens and pigs. The sewage sample, in which six strains presented the chuA gene, five the yjaA gene and two the TspE4.C2 fragment, was plotted near the human sample (Figure 3).

The discrimination power of the phylogenetic groups A, B1, B2 and D was also tested using CA (Figure 4). According to this analysis, the bidimensional representation of the phylo-groups relative abundance can explain 99.1% of the total inertia, being the horizontal axis responsible for 82.54% of it. This analysis revealed that the phylo-group distribution among cows, goats and sheep, which presented a predominance of strains of the B1 group, was similar. Humans, chickens and pigs remained separated. E. coli strains isolated from two Rivers, Jaguari and Sorocaba, located in the State of São Paulo, Brazil, and previously analyzed by Orsi et al. [23], were also included in this CA analysis (data not shown).

The strain composition of the Jaguari River included 42 strains of group A, 13 strains of group B1 and six strains of group D. The Sorocaba River included 45 strains of group A, 14 strains of group B1, one strain of group B2 and eight strains of group D. The strains distribution among the phylo-groups, from both rivers, was similar to the one observed for chickens and pigs. The sewage sample was also included in this CA and once again, this sample was similar to humans (Figure 4).

The results obtained with the classifier tools BLR and PLS-DA using the genetic markers are summarized in Table 5. The separation between E. coli strains of omnivorous and herbivorous mammals presented the lowest classification error rate (17% on average), while the highest classification error rate (25% on average) was observed between E. coli strains of humans and non-humans. Both classifier tools demonstrated that the chuA and the yjaA genes were more informative to discriminate between E. coli strains of human and non-human sources (data not shown). The PLS-DA tool showed that

| Phylogenetic subgroup | Human | Cow | Chicken | Pig | Sheep | Goat |
|-----------------------|-------|-----|---------|-----|-------|------|
| A0                    | 0     | 12  | 7       | 4   | 4     | 1    |
| A1                    | 38    | 2   | 3       | 17  | 0     | 2    |
| B1                    | 8     | 29  | 2       | 9   | 20    | 13   |
| B2_2                  | 5     | 0   | 1       | 2   | 0     | 0    |
| D1                    | 26    | 4   | 0       | 5   | 3     | 0    |
| D2                    | 10    | 3   | 0       | 2   | 2     | 0    |
| Total                 | 94    | 50  | 13      | 39  | 29    | 16   |

Table 1: Distribution of the E. coli phylogenetic subgroups among the hosts analyzed.
the \textit{yjaA} gene and the TspE4.C2 DNA fragment were more informative to discriminate between \textit{E. coli} strains of herbivorous and omnivorous mammals. The error rate for BLR and PLS-DA was higher in the prediction of human than in non-human samples (data not shown). However, when the feeding habit of mammals was considered in the separation, the error rate for both tools was higher in the prediction of the herbivorous samples.

**Discussion and Conclusions**

This study demonstrated that phylogenetic subgroup, group and genetic markers distribution are not randomly distributed among the hosts analyzed. The results showed a similarity between the \textit{E. coli} population structure of humans and pigs (omnivorous mammals) and of cows, goats and sheep (herbivorous mammals). Humans and pigs exhibited the highest diversity indexes, while goats and sheep exhibited the lowest ones. Using the simulations of the EcoSim software [24], it was possible to conclude that the diversity indexes are significantly different among the herbivorous and omnivorous mammals. The Pianka's similarity index showed that the human sample was more similar to the pig sample (88.3% of overlap). Cows, goats and sheep also presented a high overlap (96% on average), while chickens presented the lowest values.

Cows, goats and sheep are ruminant mammals which differ in many gut characteristics from other animals. Humans and pigs present common gut characteristics because they are monogastric animals (reviewed in [25]). Besides the gut characteristics, the diet of the host appears to have selected the phylo-group profile in the Brazilian mammals analyzed in this work. Omnivorous mammals presented a prevalence of phylo-group A, while the herbivorous mammals presented a prevalence of phylo-group B1. Previous research by Gordon and Cowling [10] revealed a different result from ours, identifying a prevalence of strains of phylo-group B2 among herbivorous and omnivorous mammals and a prevalence of phylo-group B1 among birds and carnivorous mammals, which supports their hypothesis of geographic effects in the \textit{E. coli} population structure among hosts. However, they also concluded that phylo-groups A and B1 are "generalists" and B2 and D are "specialists", which is in agreement with our data since strains of group B1 were found in all the hosts analyzed, followed by subgroups A\textsubscript{0} and A\textsubscript{1}. On the other hand, subgroup B2\textsubscript{3} was present only in the human sample. Therefore, our results suggest that B2 strains, especially subgroup B2\textsubscript{3}, could be a good indicator of human feces contamination.

Group B1 was prevalent among the herbivorous hosts. However, this phylo-group is not a promising indicator of herbivorous feces contamination because it was found in all the hosts analyzed, and, apparently, most \textit{E. coli} strains that are able to survive in the environment, belong to this group [12]. According to our data, the distribution analysis of phylo-groups A and D is a powerful discriminating tool since both groups presented a considerable contribution to the Chi-square values (data not shown).

The \textit{chuA} and \textit{yjaA} genes were rarely found in strains of cows, goats and sheep but were commonly found in human, chicken and pig strains. Sobieszczakiewska [26] showed that 95.5\% of the enteroaggregative \textit{E. coli} strains carried the \textit{chuA} gene, which encodes for a haem receptor. Strains belonging to group B2 were not found in cows, goats and sheep. Other studies have demonstrated that B2 and D strains are usually more pathogenic than A and B1 strains [16,17,27,28]. In fact, verocytotoxin-producing \textit{E. coli}, like O157:H7, belongs to group D [29] and cattle are the main reservoirs of this pathogen. The prevalence of groups B2 and D and of the \textit{chuA} and \textit{yjaA} genes in humans and pigs might suggest that fecal contamination by these animals can present a high risk of extra-intestinal pathogenic \textit{E. coli}. Thus, the correct identification of this kind of fecal contamination can also be useful to the appropriate management of environmental pollution.

**Table 2: Distribution of the \textit{E. coli} genetic markers among the hosts analyzed**

| Genetic marker | Human | Cow | Chicken | Pig | Sheep | Goat | Total |
|----------------|-------|-----|---------|-----|-------|------|-------|
| \textit{chuA}  | 48    | 7   | 1       | 9   | 5     | 0    | 70    |
| \textit{yjaA}  | 50    | 2   | 4       | 19  | 0     | 2    | 77    |
| TspE4.C2       | 25    | 32  | 2       | 11  | 22    | 13   | 105   |

**Table 3: Shannon's and Simpson's diversity index of each host analyzed**

| Diversity index | Human | Cow | Chicken | Pig | Sheep | Goat |
|-----------------|-------|-----|---------|-----|-------|------|
| Shannon index   | 0.6598| 0.5029| 0.5025  | 0.6523| 0.412 | 0.2614|
| Simpson index   | 0.7331| 0.5944| 0.6272  | 0.7245| 0.4899| 0.3203|
Correspondence analysis is a descriptive/exploratory technique, based on Chi-square values, that allows the exploration of the structure of the data. In the three CA models performed, similar distribution patterns were observed among \textit{E. coli} strains of herbivorous mammals and among strains of omnivorous mammals. Furthermore, the CA of subgroup distribution allowed the discrimination of omnivorous mammals. Similar results were observed by Baldy-Chudzik \textit{et al.} [20]. These authors suggested that the \textit{E. coli} strains of group B1 are best adapted to herbivorous, whereas strains of group A are best adapted to omnivorous mammals. The three CA models correctly predicted the animal/human source of the external validation sample (sewage), indicating that a significant part of the \textit{E. coli} phylo-group diversity was covered by the strains database, which reveals the stability of the models. \textit{E. coli} samples from the Jaguari and Sorocaba Rivers [23] were also used to test the CA model based on phylo-group distribution. Our analysis suggested that pigs were the major source of fecal contami-

|       | Cow   | Chicken | Pig   | Sheep | Goat |
|-------|-------|---------|-------|-------|------|
| Human | 0.286 | 0.350   | 0.883 | 0.256 | 0.281|
| Cow   | -     | 0.585   | 0.566 | 0.979 | 0.936|
| Chicken | -     | -       | 0.609 | 0.414 | 0.372|
| Pig   | -     | -       | -     | 0.507 | 0.574|
| Sheep | -     | -       | -     | -     | 0.966|

**Table 4: Pairwise Pianka’s index of similarity among the hosts analyzed**

**Figure 2** Correspondence analysis using the contingency table of subgroup distribution among the hosts analyzed. Subgroups and samples that are similar fall close. Eigenvalues are 0.47575 for the horizontal axis and 0.12813 for the vertical axis. The horizontal axis is responsible for 73.85% of the total inertia and the vertical axis for 19.89%.
nation in both rivers, which is in agreement with Orsi et al. [23], confirming that the major source of fecal contamination of these rivers was non-human. Therefore, these results indicate that the CA model can be efficiently applied in the discrimination of \textit{E. coli} strains from different animal sources.

Both classifier tools (BLR and PLS-DA) and both validation methods (cross-validation and train-test) exhibited similar overall error rates for each strain separation analyzed. This way, the statistical method used did not show a significant interference in the obtained results. Excluding the chicken sample, the best classification was obtained when the \textit{E. coli} strains were separated according to the feeding habits of the hosts (omnivorous and herbivorous mammals). Although the classification error rates found could be considered high, similar error rates were observed in other BST studies [30,31].

Since it is very difficult to find host-specific strains or genetic markers [4,32], in this work we propose a new approach to identify the animal source of fecal contamination in water systems. This approach is based on the specificity of the \textit{E. coli} population structure instead of host-specific strains. Geographic variation of the \textit{E. coli} population structure was reported in the literature [10,32] and since the relative abundance of phylo-groups among hosts can be easily characterized, this approach can be implemented in different regions of the world as a supplementary bacterial source tracking tool. Although our data is consistent in showing the potential applicability of this approach, we are aware that there might be some limitations due to the limited number of fecal pollution sources analyzed.

**Methods**

The present study has been approved by the Research Ethics Committee of the State University of Campinas School of Medical Sciences.

**Escherichia coli Strains**

Two hundred and forty one strains of \textit{E. coli} were isolated (collected with sterile swabs) from fecal samples of a vari-

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**Figure 3** Correspondence analysis using the contingency table of phylogenetic group distribution among the hosts analyzed. Phylo-groups and samples that are similar fall close. Eigenvalues are 0.33431 for the horizontal axis and 0.06708 for the vertical axis. The horizontal axis is responsible for 82.54% of the total inertia and the vertical axis for 16.56%.
ety of hosts (Table 6). Each strain was isolated from a single animal. These strains were used to build the calibration set for further statistical analysis.

Twelve sewage strains isolated by CETESB (Table 6), the organization responsible for the control of environmental pollution, sewage, and water quality in the State of São Paulo, Brazil, were used as the external validation set. The sewage samples were collected in 2008 at the Jesus Neto sewage treatment plant.

The strains were isolated as described by Orsi et al. [23], with modifications. Samples were analyzed using the membrane filter technique with modified mTEC agar (Difco) and incubated for 2 h at 35 ± 0.5°C and 22--24 h at 44.5 ± 0.2°C. Typical colonies were streaked on EMB agar (Merck). Isolated colonies were tested for citrate utilization, lactose fermentation, oxidase, l-lysine decarboxylase, motility, glucose and sucrose fermentation, tryptophan deamination, indole production, urea hydrolysis and sulfide production. Isolates with an E. coli profile were inoculated into LB broth at 37°C overnight. One isolated colony from each EC positive sample was selected for further analyses.

**Phylogenetic group determination**

The phylogenetic group of each strain was determined according to Clermont et al. [19], by multiplex PCR of the genes chuA and yjaA and the DNA fragment TspE4.C2. The amplification products were separated in 2% agarose gels containing ethidium bromide [33]. After electrophoresis, the gel was photographed under UV light, and the strains were assigned to the phylogenetic groups B2 (chuA+, yjaA+), D (chuA+, yjaA-), B1 (chuA-, TspE4.C2+) or A (chuA-, TspE4.C2-).

To increase the strains discrimination, subgroups or phylotypes were determined as follows: subgroup A0 (group A), chuA-, yjaA-, TspE4.C2-; subgroup A1 (group A), chuA-, yjaA+ TspE4.C2-; group B1, chuA-, yjaA-, TspE4.C2+; subgroup B22 (group B2), chuA+, yjaA+, TspE4.C2-; subgroup B23 (group B2), chuA+, yjaA+, TspE4.C2+; subgroup D1 (group D), chuA+, yjaA-, TspE4.C2-.
TspE4.C2- and subgroup D_2 (group D), chuA+, yjaA-, TspE4.C2+ [5].

**Bioinformatic and statistical analysis**

A graphic representation was used to map the occurrence of the genetic markers chuA, yjaA and TspE4.C2 in the E. coli strains isolated from the different hosts. For this, the genetic markers were scored as present/absent in each strain analyzed, and the graphic was drawn with the software Pajek v. 1.22 [http://vlado.fmf.uni-lj.si/pub/networks/pajek/]. This graphic provides a useful representation of the E. coli phylo-groups among the different hosts. It contains two sets of nodes -- genetic markers and samples -- and edges between them. An edge between two nodes means that the genetic marker was detected for a given strain.

The prevalence index (P) was calculated by dividing the number of hosts exhibiting a particular subgroup by the total number of hosts analyzed. The results were expressed as percentages [34].

The Pianka’s index was calculated to evaluate the subgroup overlap between two hosts by using the formula: $O = \frac{\sum p_j \times p_k}{\sqrt{\sum p_j^2 \times \sum p_k^2}}$, where $p_j$ and $p_k$ are subgroups proportions in the hosts $j$ and $k$, respectively. The results were expressed as percentages [35].

The Chi-square test, the Simpson’s diversity index and the Shannon’s index were performed with the BioEstat v. 5.0 software [36], using the phylogenetic subgroup data. The EcoSim software [24] was used to test the differences among the diversity indexes by using resampling. The frequencies of phylogenetic groups, subgroups and genetic markers were compared among the hosts by using the CA, which was performed by using STATISTICA 6.0 [37]. The sewage sample was used to challenge the CA models as an external validation sample.

The classifier tools Binary Logistic Regression (BLR) and Partial Least Squares -- Discriminant Analysis (PLS-DA) were performed with the software TANAGRA 1.4 [38]. For these analyses, the hosts were separated into humans and non-humans, human and non-human mammals, omnivorous and herbivorous mammals. The genetic markers were scored as present/absent. The cross-validation of these analyses was carried out by using five repetitions and ten fold parameters, and the train-test was carried out using 70% of the samples as a training set and ten repetitions of assessment.

**Authors’ contributions**

CC and LMMO conceived and designed the study. CC performed the experiments, the statistical analysis and wrote the manuscript. MMP performed the bioinformatic analysis. NCS, TATG and LAA isolated the majority of the E. coli strains used in the work. MIZS and EMH participated in the discussion of the experimental results. All authors read and approved the final manuscript.

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**Table 5: Classification error rates obtained by validation of supervised learning classifier tools (BLR and PLS-DA)**

| E. coli strains sources          | Classifier tool | Overall cross-validation error rate | Overall test error rate |
|----------------------------------|-----------------|------------------------------------|------------------------|
| Humans and non-humans            | BLR             | 22.50%                             | 24.93%                 |
|                                  | PLS-DA          | 25.33%                             | 27.53%                 |
| Humans and non-humans mammals    | BLR             | 22.09%                             | 22.03%                 |
|                                  | PLS-DA          | 22.09%                             | 22.75%                 |
| Omnivorous and herbivorous mammals| BLR             | 16.57%                             | 16.67%                 |
|                                  | PLS-DA          | 18%                                | 17.39%                 |

The classification was carried out between human and animal samples, between humans and non-humans mammals and between omnivorous and herbivorous mammals.

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**Table 6: Source and number of E. coli strains used in this study**

| Source     | Number of Strains | References                  |
|------------|-------------------|-----------------------------|
| Human      | 94                | Gomes et al. [39]           |
| Cow        | 50                | Vicente et al. [40]         |
| Chicken    | 13                | Silveira et al. [41]        |
| Pig        | 39                | Isolated according to Vicente et al. [40] |
| Goat       | 16                | Isolated according to Vicente et al. [40] |
| Sheep      | 29                | Isolated according to Vicente et al. [40] |
| Sewage     | 12                | Isolated by CETESB according to Orsi et al. [23] |
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