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Copper Resistance in Aerobic Intestinal Bacteria from Children with Different Levels of Copper-Exposure

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Abstract

Copper is an environmental element, essential for life, to which humans are exposed by inhalation, hand-to-mouth contamination, or ingestion of food and water. In this work, bacterial copper susceptibility (amount of copper able to inhibit bacterial growth) of aerobic bacteria from the intestinal microbiota of healthy children in Spain was explored. To establish the possible effect of children's exposure to copper in the selection of copper-resistant organisms, the prevalence of copper-resistance among bacteria isolated in stool samples of 233 children belonging to the INMA cohort of the Spanish Project for Environment and Childhood Research was studied. Stool samples were seeded into Szybalsky-type agar plates containing specific culture media and a gradient of copper sulphate (< 0.68 - 2.05 mM). Culture media was suitable for growth of Gamma-Proteobacteria (mostly Enterobacteriaceae), *Enterococcus* and *Staphylococcus*. Colonies growing at intermediate (IR: < 0.68 - 1.36 mM) and/or higher copper concentrations (HR: 1.36 - 2.05 mM) were characterized by MaldiTOF assays. Sixty different species of copper-resistant organisms were detected. For *Enterobacteriaceae*, HR colonies were detected in 64.5% of seeded fecal samples, mostly corresponding to genus *Escherichia* (77% of positive samples); *Enterobacter* (15%), *Citrobacter* (13%), and *Klebsiella* (4.7%). However, *Escherichia coli* populations have a significantly lower proportion of high copper resistant colonies (49%) than the ensemble of *Klebsiella-Enterobacter-Citrobacter* colonies (77%). In HR-colonies of *Firmicutes*, *Enterococcus genus* was found in 97.3% of fecal samples, predominantly *E. faecium* (86%, of the positive Enterococcus samples), *E. faecalis* (37%), and *E. hirae* (6.9%). No significant correlation was found between counts of HR-colonies and the copper concentrations found in dry hair of the children studied (10 - 30 mcg/g). Copper-resistant populations in the children's intestine might have evolved in the copper-rich external environment.

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Introduction

Copper, an essential micronutrient for most plant and animal species, is involved in the function of several enzymes and other proteins, and needed in a wide range of metabolic processes; conversely, high concentrations can be detrimental for health[1]. However, the clinical consequences of both low and high copper-levels in the human body are rarely observed, but it should be noted that the possible effects on human microbiome diversity have scarcely been explored[2].

Human exposure to copper mainly occurs through the ingestion of food. The most significant sources of the dietary copper are yeast breads, white potatoes, tomatoes, cereals, beef, liver, seafood (particularly shellfish), dried beans and lentils[3]. Also copper reaches humans by drinking water and inhalation of polluted air (linked to occupational exposures)[4]. Natural ingredients in the normal Western diet provide approximately 2 to 4 mg copper per day; about 0.6 to 1.6 mg is absorbed, from 0.5 to 1.2 mg is excreted in the bile, 0.1 to 0.3 mg passes directly into the bowel, and 0.01 to 0.06 mg appears in the urine[5]. Much of the copper that is absorbed is later excreted in the bile so that more than 90 percent of ingested copper is found in feces[6]. Note that natural food exposure might be eventually be increased by GRAS ("Generally Recognized as Safe"), including cuprous iodide in table salt, as nutrients and/or dietary supplements, as antiseptic in paper and paperboard products used in food packaging, and growth promoters in foodborne animals[7].

Dietary supplements including copper in farm animals might modify the bacterial microbiota, frequently reducing Gram-negative facultative (coliform) organisms[8]. In fact poultry coliforms (Enterobacteriaceae, Proteobacteria) are more susceptible to copper than gram positives as *Lactobacillus* (Firmicutes)[9]. Early studies suggested a relative tolerance of lactobacilli and *Escherichia coli* to copper as dietary supplement[10].

The possible effects of copper on the normal microbiome were not listed among the biological effects of copper human exposure[4]. Most importantly, copper-resistance genes are frequently located in the same genetic platforms as genes encoding resistance to other metals (zinc, mercury, arsenic) and antibiotic resistance genes, and therefore the conditions favoring copper-resistance in the individual might influence antibiotic-resistance and vice versa[11,12].

The aim of this work is to investigate the frequency of copper-resistance in bacterial populations from the major facultative Proteobacteria and Firmicutes taxa in the intestine of healthy children with different documented levels of copper in dry hair. We studied the prevalence of bacterial copper-resistance in fecal samples of 233 children belonging to the INMA cohort. Samples were seeded in selective culture media containing gradients of intermediate (< 0.68 - 1.36 mM) or high (1.36 - 2.05 mM) copper concentrations. This range of concentrations, lower than are generally studied, permits exploration of the effects of copper when it starts influencing growth of bacterial populations. This semi-quantitative approach allowed a complete detection of the target intestinal population able to form colonies in a continuum of concentrations, thus revealing different levels of copper-resistance. With this technique we reached our objectives addressed to obtain: 1) the proportion of intestinal samples containing the different copper-resistant species; 2) the frequency of copper-resistance among isolates of different bacterial species, and 3) the correlation between copper exposure and the density or frequency of copper resistant bacteria.

There was an almost absolute absence of data concerning the levels of exposure of normal individuals to copper under the standard conditions of developed countries, and the effects that such exposure produce in the human intestinal microbiota. Our research was focused on ascertaining this correlation by measuring changes in the proportion of copper-resistant bacteria (selection for resistance) in children with different levels of copper resistance. At the same time, our work for the first time documents the frequency of copper-resistant bacteria in clinically-relevant bacteria of the intestinal microbiota of healthy children.

Material and Methods

Study population and sample collection

The prevalence of copper-resistance in fecal samples was studied in 233 healthy children (1 - 8 years old), randomly selected from different Spanish towns and regions (Guipuzkoa, Sabadell and, Valencia), belonging to the cohort of Spanish Project for Environment and Childhood Research (INMA, http://www.proyectoinma.org/). The study was approved by the regional ethical committees of each regional cohort and all the parents of the children signed written informed consent before beginning the study. A total of 166 fecal samples (at least 10 g each, obtained by natural defecation) were obtained in Sabadell and Guipuzcoa, applying the MetaHit sampling protocol[13]. In children from Valencia, 67 rectal swabs (Conda Laboratories, Torrejon, Madrid, Spain) samples were obtained. In all the cases, samples were immediately frozen and transported to the recipient laboratory where they were stored at -40ºC.

Chemical analysis of copper concentration in children's hair

Chronic exposure to copper was evaluated in 75 children by the standard procedure in our group for measuring the metal concentration in the hair[14]. A minimum of 10 mg of hair from occipital scalp was collected for each child, placing samples in a plastic bag and storing them at room temperature until their dispatch. Copper concentrations in hair were obtained by using an atomic absorption spectrometer (AMA-254 analyzer from the Leco Corp. Praha, Czech Republic) at the Environmental Chemistry Department (IIQAB) of the CSIC (Barcelona, Spain).

Stool samples

Stool samples included feces (solid fecal material, fecal samples) and rectal swabs. For feces, one gram was homogenized by vortexing into 5 ml of sterile saline (9 g of sodium chloride salt per litre). One-tenth dilution was performed in saline, and 200 µL were seeded (rolling beads) into the copper-containing culture media. This dilution was plated on Petri square (12 x 12 cm, 144 cm^2) gradient plates, containing culture agar media with a copper sulfate gradient ($CuSO_4*5H_2O$) ranging from 0 to 512 µg/ml (0 - 2.05 mM). McConkey Agar medium (Becton Dickinson BBLTMMcConkey Agar, Ref. 211387) was used for Enterobacteriaceae (Proteobacteria) recovery, M-*Enterococcus* Agar (Becton Dickinson DifcoTM m-*Enterococcus* Agar, Ref. 274620) for selected aerobic Firmicutes recovery, and Mannitol Salt Agar (Conda, Ref. 1062.00). Due to the expected differences in growth rates between these organisms, McConkey plates were incubated at 37ºC for 24 h, and for M-*Enterococcus* and

Mannitol Salt agar (MSA) plates for 48 h.

For rectal swabs, the procedure was as following: the swab was suspended in 3 ml of Brain Heart Infusion (Conda, Ref 1400.00), and incubated for 24 h at 37ºC. From this culture, 1.5 ml was centrifuged at 6,000 rpm for 6 minutes, and the pellet was re-suspended in 1 ml saline, and the optical density adjusted at 0.5 McFarland. 200 µL of this suspension were seeded (rolling beads) into the copper-containing gradient plates (see above paragraph), and incubated at 37ºC for 24 h for McConkey plates, and for 48 h for M-*Enterococcus* and Mannitol Salt agar (MSA) agar plates.

Categorization of bacterial copper susceptibility

Due to the absence of well-defined cut-offs for establishing susceptibility or resistance to heavy metals (including copper) in natural bacterial isolates, and the expected wide gradient distribution of copper concentrations in nature, the evaluation of the inhibitory effect of copper was done as follows: the surface of the square plate was divided in three sectors, corresponding to copper concentrations of < 170 µg/ml (< 0.68 mM), 170 - 340 µg/ml (0.68 - 1.36 mM) and 340 - 512 µg/ml (1.36 - 2.05 mM). All colonial morphotypes growing in the different sectors of the plates were considered for identification and differential counting. In cases of uncountable or very scarce growth along the gradient plate, the assay was repeated with a higher or less diluted inoculum to be able to detect individual colonies. This number of colonial morphotypes is referred to along the manuscript as "number of isolates". Colony counts were performed in all three sectors of the plate. For each sample the total number of colonies in the sectors of the gradient plate was counted. The density (colony counts/ml of sample) of the bacteria belonging to the aerobic taxa Gamma-Proteobacteria and Firmicutes was plotted against the copper levels measured in the hair sample of the corresponding child.

Bacterial colony species identification was performed with a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) method, using the Microflex LT mass spectrometer (Bruker Daltonics GmbH, Leipzig, Germany) with the FlexControl 3.0 and MALDI BioTyper 2.0 and 3.0 software programs.

Data analysis and statistics

For statistical analysis we used the software provided in the Social Science Statistics web site (www.socistatistics.com). When the p-value is not specifically presented, significant differences are considered if $p < 0.01$. The Pearson correlation (r) was obtained to analyze the plots of colony counts/ml in all three sectors of the gradient plates versus the copper levels measured in the hair sample of the corresponding child.

Results

Copper resistance in intestinal Gamma-Proteobacteria

A detailed account of all bacterial species of Gamma-Proteobacteria with copper-resistant bacteria is shown in (Table 1). In the following paragraphs, the analysis of the frequency of recovery of these bacteria in stool samples and fecal swabs, and the proportion of copper resistant organisms in the different taxa is presented.

Table 1: Identified species containing organisms with high resistance to copper, (MIC 340 - 512 µg/ml, or 1.36 - 2.05 mM) in the INMA cohort (Spain).

Phylum	Genus	Species	Number
Proteobacteria	*Escherichia* (211)	*E. coli* (210)	105
		E. hermanii (1)	0
	Enterobacter (20)	*E. asburiae* (2)	2
		E. cloacae (18)	15
	Citrobacter (19)	*C. braakii* (5)	2
		C. freundii (13)	11
		C. youngae (1)	1
	Klebsiella (14)	*K. oxytoca* (9)	4
		K. pneumoniae (4)	3
		K. variicola (1)	1
	Raoultella (6)	*R. ornithinolytica* (3)	1
		R. planticola (3)	3
	Pseudomonas (5)	*P. fragi* (1)	1
		P. monteilli (1)	1
		P. plecogiossicida (1)	1
		P. putida (2)	2
	Acinetobacter (2)	*A. calcoaceticus* (1)	1
		A. junii (1)	1
	Salmonella (1)	*Salmonella sp.* (1)	1
	Kluyvera (1)	*K. cryocrescens* (1)	1
	Hafnia (1)	*H. alvei* (1)	1
	Comamonas (1)	*C. kerstersii* (1)	1
Firmicutes	*Enterococcus* (397)	*E. faecium* (246)	196
		E. faecalis (101)	72
		E. hirae (15)	11
		E. durans (14)	8
		E. casseliflavus (7)	2
		E. avium (6)	3
		E. mundtii (3)	1
		E. gallinarum (2)	2
		E. gilvus (1)	0
		E. raffinosus (1)	0
		E. thailandicus (1)	0
	Streptococcus (21)	*S. constellatus* (1)	0
		S. equinus (1)	0
		S. pasteurianus (3)	1
		S. salivarius (16)	1
	Lactobacillus (11)	*L. paracasei* (6)	1
		L. plantarum (3)	2
		L. sakei (2)	0
	Pediococcus (8)	*P. pentosaceus* (8)	6
	Aerococcus (1)	*A. viridans* (1)	1
	Staphylococcus (67)	*S. aureus* (18)	5
		S. epidermidis (22)	7
		S. hominis (10)	3
		S. arlettae (3)	1
		S. saprophyticus (3)	3

		S. xylosus (3)	3
		S. cohnii (2)	1
		S. equorum (2)	2
		S. haemolyticus (2)	2
		S. pasteuri (1)	1
		S. succinus (1)	1

Proportion of intestinal stool samples containing the different copper-resistant species (Table 2)

Fecal samples: Colonies highly resistant to copper (MIC 340 - 512 µg/ml, or 1.36 - 2.05 mM) were detected in 107 cases from 166 stool samples (64.5%), corresponding to species of the genus *Escherichia* (in 77.6% of the samples, 83/107), *Enterobacter* (15.0%, 16/107), *Citrobacter* (13.1%, 14/107), *Klebsiella* (4.7%, 5/107), *Pseudomonas* (3.7%, 4/107), *Raoultella* (1.9%, 2/107), *Acinetobacter* (1.9%, 2/107), *Salmonella* (0.9%, 1/107), *Kluyvera* (0.9%, 1/107), *Hafnia* (0.9%, 1/107), *Comamonas* (0.9%, 1/107), and other non-identified organisms (3.7%, 4/107). In 58.9% (63/107) of the samples only one resistant species was detected, but 28.0% (30/107) had two resistant species, 9.3% (10/107) three resistant species and 1.9% (2/107) of the samples contained four highly resistant species. Colonies with growth in a copper concentration of 170 - 340 µg/ (0.68 - 1.36 mM, intermediate section of the Petri gradient plate) but not beyond (note that these samples might also contain different colonies from other species growing at higher concentrations) were found in most of the samples (90.4 %, 150/166). They were identified as species of the genera *Escherichia* (in 90.7% of the samples, 136/150), *Citrobacter* (12.0%, 18/150), *Enterobacter* (10.7%, 16/150), *Klebsiella* (5.3%, 8/150), *Raoultella* (2.7%, 4/150), *Acinetobacter* (1.3%, 2/150), *Salmonella* (0.7%, 1/150), *Kluyvera* (0.7%, 1/150), *Hafnia* (0.7%, 1/150), *Comamonas* (0.7%, 1/150), and non-identified organisms (4.7%, 7/150).

Table 2: Gram negative (Gamma-Proteobacteria) isolates obtained in McConkey agar media supplemented with copper at high (340-512 µg/ml) and intermediate concentrations (170-340 µg/ml). Proportions (per sample and per species) from feces and rectal swabs are detailed in the table. Note that the same sample might contain different resistant bacteria species (the total of positive samples per microorganism might exceed the total number of positive samples).

	Per sample (166 feces, 67 swabs)									**Per bacterial isolates (288 from feces, 140 from swabs)**							
	Growing at high Cu++				**Growing at intermediate Cu++**					**Growing at high Cu++**				**Growing at intermediate Cu++**			
	Feces		**Swabs**		**Feces**		**Swabs**			**Feces**		**Swabs**		**Feces**		**Swabs**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**		**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Samples with R strains	107	64.5	65	95.6	150	90.4	65	95.6	**Resistant isolates**	163	56.6	132	94.3	271	94.1	140	100.0
Escherichia coli	83	77.6	49	75.4	136	90.7	51	78.5	***Escherichia coli***	105	64.4	71	53.8	199	73.4	77	55.0
Escherichia hermanii	0	0.0	1	1.5	0	0.0	1	1.5	***Escherichia hermanii***	0	0.0	1	0.8	1	0.4	1	0.7
Enterobacter cloacae	14	13.1	17	26.2	14	9.3	18	27.7	***Enterobacter cloacae***	15	9.2	23	17.4	15	5.5	24	17.1
Enterobacter aerogenes	0	0.0	2	3.1	0	0.0	2	3.1	***Enterobacter aerogenes***	0	0.0	2	1.5	0	0.0	2	1.4
Enterobacter asburiae	2	1.9	1	1.5	2	1.3	1	1.5	***Enterobacter asburiae***	2	1.2	1	0.8	2	0.7	1	0.7
Citrobacter freundii	11	10.3	2	3.1	13	8.7	2	3.1	***Citrobacter freundii***	11	6.7	3	2.3	13	4.8	3	2.1
Citrobacter braakii	2	1.9	1	1.5	4	2.7	1	1.5	***Citrobacter braakii***	2	1.2	3	2.3	4	1.5	3	2.1
Citrobacter youngae	1	0.9	0	0.0	1	0.7	0	0.0	***Citrobacter youngae***	1	0.6	0	0.0	1	0.4	0	0.0
Klebsiella pneumoniae	2	1.9	13	20.0	2	1.3	14	21.5	***Klebsiella pneumoniae***	3	1.8	15	11.4	3	1.1	16	11.4
Klebsiella oxytoca	4	3.7	3	4.6	5	3.3	3	4.6	***Klebsiella oxytoca***	4	2.5	3	2.3	7	2.6	3	2.1
Klebsiella variicola	1	0.9	2	3.1	1	0.7	2	3.1	***Klebsiella variicola***	1	0.6	2	1.5	1	0.4	2	1.4
Kluyvera cryocescens	1	0.9	0	0.0	1	0.7	0	0.0	***Kluyvera cryocescens***	1	0.6	0	0.0	1	0.4	0	0.0
Hafnia alvei	1	0.9	1	1.5	1	0.7	1	1.5	***Hatnia* spp**	1	0.6	1	0.8	1	0.4	1	0.7
***Proteus* spp**	0	0.0	1	1.5	0	0.0	1	1.5	***Proteus* spp**	0	0.0	1	0.8	0	0.0	1	0.7
***Morganella* spp**	0	0.0	1	1.5	0	0.0	1	1.5	***Morganella* spp**	0	0.0	1	0.8	0	0.0	1	0.7
***Raultella* spp**	2	1.9	0	0.0	4	2.7	0	0.0	***Raultella* spp (4 species)**	4	2.5	0	0.0	6	2.2	0	0.0

Comamonas kerstersii	1	0.9	0	0.0	1	0.7	0	0.0	***Comamonas kerstersii***	1	0.6	0	0.0	1	0.4	0	0.0
Salmonella spp	1	0.9	0	0.0	1	0.7	0	0.0	***Salmonella spp***	1	0.6	0	0.0	1	0.4	0	0.0
Acinetobacter junii	1	0.9	0	0.0	1	0.7	0	0.0	***Acinetobacter junii***	1	0.6	0	0.0	1	0.4	0	0.0
Acinetobacter calcoaceticus	1	0.9	0	0.0	1	0.7	0	0.0	***Acinetobacter calcoaceticus***	1	0.6	0	0.0	1	0.4	0	0.0
Pseudomonas spp	4	3.7	0	0.0	4	2.7	0	0.0	***Pseudomonas spp (4 species)***	5	3.1	0	0.0	5	1.8	0	0.0
Non-identified	4	3.7	4	6.2	7	4.7	4	6.2	***Non-identified***	4	2.5	4	3.0	7	2.6	4	2.9

Rectal swabs: Using enriched cultures from rectal swabs, the recovery of colonies highly resistant to copper was increased in relation to that obtained with fecal samples (65/68; 95.6% vs in 107/166; 64.5%). Colonies corresponded to *Escherichia* (in 76.9% of the samples, 50/65), *Enterobacter* (30.8%, 20/65), *Klebsiella* (27.7%, 18/65), *Citrobacter* (4.6%, 3/65), *Hafnia* (1.5%, 1/65), *Morganella* (1.5 %, 1/65), and Proteus mirabilis (1.5%, 1/65), and non-identified (6.2%, 4/65). Intermediate copper-resistant colonies were found in 65 out of 68 samples (95.6%), containing *Escherichia* (80%, 52/65, *Enterobacter* (32.3%, 21/65), *Klebsiella* (29.2%, 19/65), *Citrobacter* (4.6%, 3/65), *Hafnia, Morganella* and *Proteus* (1.5 %, 1/65 each one), and non-identified (6.2%, 4/65). Species of *Raoultella, Salmonella, Acinetobacter, Pseudomonas* or *Comamonas* were not recovered from rectal swabs.

Frequency of copper-resistance among isolates of different bacterial species and genera (Table 2)

Fecal samples: The frequency of strains (all colonial morphotypes in all stool fecal samples considered) with high copper-resistance varied among the different bacterial genera; in *Escherichia* (105 Cu-R among 211 *Escherichia* colonies, 105/211, 49.8%), in *Enterobacter* (17/20, 85%); in *Citrobacter* (14/19, 73.68%), in *Klebsiella* (8/14, 57.14%), *Raoultella* (4/6, 66.7%), *Pseudomonas* (5/5), *Acinetobacter* (2/2), *Salmonella* (1/1), *Kluyvera* (1/1), *Hafnia* (1/1), *Comamonas* (1/1), and non-identified 4/9). In strains with high copper-resistance, *Escherichia* strains were less frequently resistant than *Citrobacter* ($p < 0.05$), *Enterobacter* ($p < 0.05$), and *Klebsiella* ($p = 0.05$). Considering all 70 isolates of non-*E. coli* predominantly environmental species, high copper resistance reaches 77.14%, and the proportion of copper-resistance among all 211 *E. coli* (predominantly enteric) was significantly lower, 48.81 % ($p < 0.001$). The frequency of strains able to grow at intermediate copper concentrations (in a proportion, also in higher ones, see preceding paragraph) were: *Escherichia* (200/211, 94.8%), *Enterobacter* (18/20, 90%), *Klebsiella* (11/14, 78.57%) and *Citrobacter, Acinetobacter, Comamonas, Kluyvera, Pseudomonas, Raoultella, Salmonella* and non-identified (100%).

Rectal swabs: In the case of rectal swabs, the frequency of strains with high copper-resistance was: *Escherichia* (72/77, 93.50%), *Klebsiella* (18/19), 94.74%, and *Enterobacter* (13/13, 100%), *Citrobacter, Hafnia, Morganella*, and *Proteus* (100%). In summary, in the case of *E. coli* (211 strains) a mean of 94.8% of the strains (colony morphotypes) were able to grow in the range 170 - 340 µg/ml, and a mean of 49.8% were growing at higher copper concentrations (340 - 512 µg/ml). For *Enterobacter, Citrobacter*, and *Klebsiella*, means of 90, 100, 78.57%, respectively survived at the range 170 - 340 µg/ml, and means of 85, 73.68 and 57.14% respectively survived at 340 - 512 µg/ml.

Copper resistance in intestinal Firmicutes

A detailed account of all bacterial species of Firmicutes with copper-resistant bacteria is shown in Table 1. In the following paragraphs, the analysis of the frequency of recovery of these bacteria in stool samples and fecal swabs, and the proportion of copper resistant organisms in the different taxa is presented.

Proportion of intestinal samples containing the different copper-resistant species (Tables 3a, 3b)

Fecal samples: In M-Enterococcus Agar, at *high copper concentrations* (340 - 512 µg/ml, or 1.36 - 2.05 mM), resistant colonies were identified in 149 out of 166 fecal samples (89.8%). *Enterococcus* genus was found in 145 out of 149 samples (97.3%). Within *Enterococcus*, the predominant species were *E. faecium* (86.2%, 125/145 positive *Enterococcus* samples), *E. faecalis* (37.24%, 54/145), *E. hirae* (6.9%, 10/145) and *E. durans* (5.5%, 8/145). Other resistant Enterococcus species were: *E. avium* (6.9%), *E. casseliflavus* (1.4%), *E. gallinarum* (1.4%) and *E. mundtii* (0.7% of *Enterococcus*). Other resistant species were *Pediococcus pentosaceus* (4% of samples), *Lactobacillus sp* (2%), *Streptococcus sp* (1.3%), *Aerococcus viridans* (0.7%). In M-Enterococcus plates, 27.5% (41/149) of these samples showed only one resistant species, 34.9% (52/149) had two resistant species, 19.5% (29/149) three resistant species, 14.8% (22/149) four resistant species and 0.7% (1/149) five resistant species of Firmicutes. In MSA Agar plates, 40 out of 166 stool samples (24.1%) presented highly resistant isolates, and 27 out of 40 (67.5%) corresponded to *Staphylococcus* genus. Within *Staphylococcus*, eleven different species were identified: *S. epidermidis* (25.9%, 7/27 positive *Staphylococcus* samples), *S. aureus* (18.5%), *S. hominis* (11.1%), *S. saprophyticus* (11.1%), *S. haemolyticus* (7.4%), *S. equorum* (7.4%), *S. xylosus* (7.4%), *S. succinus* (3.7%), *S. pasteuri* (3.7%), *S. cohnii* (3.7%), and *S. arlettae* (3.7% of the total of *Staphylococcus*). *Lactobacillus sp* was identified in 7 samples (17.5%, 7/40), *Aerococcus viridans* in 1 sample (2.5%, 1/40) and non-identified microorganisms in 9 samples (22.5%, 9/40). In MSA plates, 70% (28/40) of the samples with resistant isolates contained 1 resistant isolates.

In M-*Enterococcus* Agar at *intermediate copper concentrations* colonies were recovered in 163 of 166 stool samples studied (98.2%). *Enterococcus* genera were found in 158 of 163 samples (96.9%). Within the genus *Enterococcus*, the species found were *E. faecium* (85.4%, 135/158), *E. faecalis* (45.6%, 72/158), *E. hirae* (6.96%, 11/158) and *E. durans*

(6.96%, 11/158). *E. casseliflavus* (n = 5), *E. avium* (2.1%), *E. mundtii* (1.9%), and *E. gallinarum* (1.3%). *Lactobacillus sp* was found in 7/163 total samples (4.3%), *Pediococcus pentosaceus* (4.3%), *Streptococcus* sp (3.1%), and *Aerococcus viridans* (0.6%). In MSA Agar plates, 73 of 166 stool samples (44.0%) presented bacterial growth at high copper concentrations, and 46 of these 73 (58.9%) were positive for *Staphylococcus*. The species identified inside the genus *Staphylococcus* were: *S. epidermidis* (39.1%, 18/46), *S. aureus* (19.6%, 9/46), *S. hominis* (17.4%), *S saprophyticus* (6.5%), *S. haemolyticus* (4.3%), *S. xylosus* (4.3%), *S. equorum* (4.3%), *S. succinus* (2.2%), and *S. pasteuri* (2.2%). Two species of *Lactobacillus* were identified in 7 samples (9.6%, 7/73), and *Aerococcus* in 1 sample (1.4%, 1/73). Two resistant isolates were detected in 25% (10/40) of samples.

Rectal swabs: In the case of enriched rectal swabs, at *high copper concentrations*, in M-*Enterococcus* Agar, 58 of the 67 samples (86.57%) contained high copper-resistant strains and 91.38% (53/58) had *Enterococcus* strains. Inside the genus *Enterococcus, E. faecium* accounted for 69.81% (37/53) *E. faecalis* for 39.6% (21/53), *E. hirae* for 9.4% (5/53), and *E. durans* for 1.9% (1/53). Only 13 samples (19.1%) were positive in MSA Agar in rectal swabs for highly resistant bacteria, corresponding in a 76.9% (10/13) to *Staphylococcus* species; *S. aureus* (n = 5), *S. epidermidis* (n = 3), *S. warneri* (n = 1) and *S. hominis* (n = 1). Non identified species were found in 30.8% (4/13) of the rectal swabs. A lower proportion of high copper-resistant isolates per sample were identified in the rectal swabs if compared with stool samples in both M-*Enterococcus* and MSA culture media, the percentages being of one Cu-resistant isolate per sample 46.55 (27/58) and 61.54% (8/13); two resistant isolates per sample in 43.1 (25/58) and 30.76% (4/13) and three resistant isolates were identified in 8.62 (5/58) and 0.0%, respectively).

Swab enriched samples inoculated in M-*Enterococcus* agar plates provided growth at *intermediate copper concentrations* in 63 out of 68 samples (94%); in 48 of them (76.2%) growth corresponded to *Enterococcus* strains. Inside *Enterococcus*, 77.1% (37/48) corresponded to *E. faecium*, 50.0% (24/48) for *E. faecalis*, 10.4 % to *E. hirae*, and 2.1 % to each one of the species *E. durans, E. avium, E. casseliflavus*, and *E. mundtii*. Copper-resistant Lactobacillus and Pediococcus were both recovered in 4.3% of the total of samples. Swab enriched samples inoculated in MSA Agar provided growth at intermediate copper concentrations in 21/67 (30.9%); *Staphylococcus* species were found in 13 of them (61.9%). Among *Staphylococcus, S. epidermidis* accounted for 53.8 %, *S. aureus* for 31.8%, and *S. hominis* and *S. warnerii* for 7.7%. *Lactobacillus sp* was found in 14.3% of the swab samples (3/21).

Table 3a: Gram positive (Firmicutes) aerobic isolates obtained in M-*Enterococcus* medium supplemented with copper at high (340 - 512 µg/ml) and intermediate concentrations (170 - 340 µg/ml). Proportions (per sample and per species) from feces and rectal swabs are detailed in the table. In italics, numbers and percentages of species inside the genus *Enterococcus*. Note that the same sample might contain different resistant bacteria species (the total of positive samples per microorganism might exceed the total number of positive samples).

	Per sample (166 feces, 67 swabs)									**Per bacterial isolates (460 from feces, 117 from swabs)**							
	Growing at high Cu++				**Growing at intermediate Cu++**					**Growing at high Cu++**				**Growing at intermediate Cu++**			
	Feces		**Swabs**		**Feces**		**Swabs**			**Feces**		**Swabs**		**Feces**		**Swabs**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**		**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Total samples																	
Samples with resistance	149	89.8	58	86.57	163	98.2	63	94.0	**Resistant isolates (R)**	325	70.7	93	79.5	410	89.1	106	90.6
With R-*Enterococcus*	145	97.3	53	91.38	158	96.9	48	76.2	**R-*Enterococcus***	295	90.8	81	87.1	365	89.0	89	84.0
E. faecium	*125*	*86.2*	*37*	*69.81*	*135*	*85.4*	*37*	*77.1*	***E. faecium***	*196*	*66.4*	*50*	*61.7*	*231*	*63.3*	*52*	*58.4*
E. faecalis	*54*	*37.2*	*21*	*39.62*	*72*	*45.6*	*24*	*50.0*	***E. faecalis***	*72*	*24.4*	*25*	*30.9*	*94*	*25.8*	*28*	*31.5*
E. durans	*8*	*5.5*	*1*	*1.89*	*11*	*7.0*	*1*	*2.1*	***E. durans***	*8*	*2.7*	*1*	*1.2*	*11*	*3.0*	*1*	*1.1*
E. hirae	*10*	*6.9*	*5*	*9.43*	*11*	*7.0*	*5*	*10.4*	***E. hirae***	*11*	*3.7*	*5*	*6.2*	*12*	*3.3*	*5*	*5.6*
E. avium	*3*	*2.1*	*0*	*0.00*	*4*	*2.5*	*1*	*2.1*	***E. avium***	*3*	*1.0*	*0*	*0.0*	*5*	*1.4*	*1*	*1.1*
E. casseliflavus	*2*	*1.4*	*0*	*0.00*	*5*	*3.2*	*1*	*2.1*	***E. casseliflavus***	*2*	*0.7*	*0*	*0.0*	*5*	*1.4*	*1*	*1.1*
E. gallinarum	*2*	*1.4*	*0*	*0.00*	*2*	*1.3*	*0*	*0.0*	***E. gallinarum***	*2*	*0.7*	*0*	*0.0*	*2*	*0.5*	*0*	*0.0*
E. mundtii	*1*	*0.7*	*0*	*0.00*	*3*	*1.9*	*1*	*2.1*	***E. mundtii***	*1*	*0.3*	*0*	*0.0*	*3*	*0.8*	*1*	*1.1*
R-*Lactobacillus* sp	3	2.0	0	0.00	7	4.3	0	0.0	**R-*Lactobacillus* sp**	3	0.9	0	0.0	8	2.0	0	0.0
R-*Aerococcus viridans*	1	0.7	0	0.00	1	0.6	0	0.0	**R-*Aerococcus viridans***	1	0.3	0	0.0	1	0.2	0	0.0
R-*Pedioc. pentosaceous*	6	4.0	0	0.00	7	4.3	0	0.0	**R-*Pedioc. pentosaceous***	6	1.8	0	0.0	8	2.0	0	0.0
R-*Streptococcus* sp	2	1.3	0	0.00	5	3.1	0	0.0	**R-*Streptococcus* sp**	2	0.6	0	0.0	7	1.7	0	0.0
R-Non-identified/ contam?	14	9.4	12	20.69	16	9.8	15	23.8	**R-Non-identified/ contam?**	18	5.5	12	12.9	21	5.1	17	14.5

Table 3b: High and intermediate resistant Firmicutes obtained in MSA agar media supplemented with copper at high (340 - 512 µg/ml) and intermediate concentrations (170 - 340 µg/ml). Counts (per sample and per species) from feces and rectal swabs are detailed in the table.

	Per sample (166 feces, 67 swabs)									Per bacterial isolates (200 from feces, 48 from swabs)							
	High Cu++ resistant				Int. Cu++ resistant					High Cu++ resistant				Int. Cu++ resistant			
	Feces		Swabs		Feces		Swabs			Feces		Swabs		Feces		Swabs	
	n	%	n	%	n	%	n	%		n	%	n	%	n	%	n	%
Samples with resistance	40	24.1	13	19.1	73	44.0	21	30.9	**Resistant isolates (R)**	48	24.0	15	31.3	149	74.5	24	50.0
With R-*Staphylococcus*	27	67.5	10	76.9	46	63.0	13	61.9	**R- *Staphylococcus***	29	60.4	11	73.3	54	36.2	17	70.8
S. epidermidis	*7*	*25.9*	*3*	*30.0*	*18*	*39.1*	*7*	*53.8*	***S. epidermidis***	*7*	*24.1*	*4*	*36.4*	*18*	*33.3*	*8*	*47.1*
S. aureus	*5*	*18.5*	*5*	*50.0*	*9*	*19.6*	*4*	*31.8*	***S. aureus***	*5*	*17.2*	*5*	*45.5*	*10*	*18.5*	*7*	*41.2*
S. saprolyticus	*3*	*11.1*	*0*	*0.0*	*3*	*6.5*	*0*	*0.0*	***S. saprolyticus***	*3*	*10.3*	*0*	*0.0*	*3*	*5.6*	*0*	*0.0*
S. haemolyticus	*2*	*7.4*	*0*	*0.0*	*2*	*4.3*	*0*	*0.0*	***S. haemolyticus***	*2*	*6.9*	*0*	*0.0*	*2*	*3.7*	*0*	*0.0*
S. hominis	*3*	*11.1*	*1*	*10.0*	*8*	*17.4*	*1*	*7.7*	***S. hominis***	*3*	*10.3*	*1*	*9.1*	*9*	*16.7*	*1*	*5.9*
S. xylosus	*2*	*7.4*	*0*	*0.0*	*2*	*4.3*	*0*	*0.0*	***S. xylosus***	*3*	*10.3*	*0*	*0.0*	*3*	*5.6*	*0*	*0.0*
S. equorum	*2*	*7.4*	*0*	*0.0*	*2*	*4.3*	*0*	*0.0*	***S. equorum***	*2*	*6.9*	*0*	*0.0*	*2*	*3.7*	*0*	*0.0*
S. succinus	*1*	*3.7*	*0*	*0.0*	*1*	*2.2*	*0*	*0.0*	***S. succinus***	*1*	*3.4*	*0*	*0.0*	*1*	*1.9*	*0*	*0.0*
S. pasteuri	*1*	*3.7*	*0*	*0.0*	*1*	*2.2*	*0*	*0.0*	***S. pasteuri***	*1*	*3.4*	*0*	*0.0*	*1*	*1.9*	*0*	*0.0*
S. warnerii	*0*	*0.0*	*1*	*10.0*	*0*	*0.0*	*1*	*7.7*	***S. warnerii***	*0*	*0.0*	*1*	*9.1*	*0*	*0.0*	*1*	*5.9*
S. cohnii	*1*	*3.7*	*0*	*0.0*	*0*	*0.0*	*0*	*0.0*	***S. cohnii***	*1*	*3.4*	*0*	*0.0*	*2*	*3.7*	*0*	*0.0*
S. arlettae	*1*	*3.7*	*0*	*0.0*	*0*	*0.0*	*0*	*0.0*	***S. arlettae***	*1*	*3.4*	*0*	*0.0*	*3*	*5.6*	*0*	*0.0*
R-*Lactobacillus sp*	7	17.5	0	0.0	7	9.6	3	14.3	**R-*Bacillus* sp**	7	14.6	0	0.0	49	32.9	3	12.5
R-*Aerococcus viridans*	1	2.5	0	0.0	1	1.4	0	0.0	**R-*Aerococcus viridans***	1	2.1	0	0.0	1	0.7	0	0.0
R-*Micrococcus luteus*	0	0.0	0	0.0	1	1.4	0	0.0	**R-*Micrococcus luteus***	0	0.0	0	0.0	1	0.7	0	0.0
R-Non-identified/ suspected contaminants	9	22.5	4	30.8	36	49.3	4	19.0	**R-Non-identified/ suspected contaminants**	11	22.9	4	26.7	44	29.5	4	16.7

Frequency of copper-resistance among isolates of different bacterial species and genera (Tables 3a and 3b)

Fecal samples: As expected, most high copper-resistant colonies (325) from stool fecal samples recovered in M-*Enterococcus* plates (MIC 340 - 512 µg/ml, or 1.36 - 2.05 mM) corresponded to the genus *Enterococcus* (90.77%, 295/325). Inside *Enterococcus*, the proportion of *E. faecium* was 66.44% (196/295), and 24.40% (72/295 = for *E. faecalis*, and 6 other Enterococcal species (*E. hirae, E. avium, E. casseliflavus, E. durans, E. gallinarum*, and *E. mundtii*) accounted for the rest (9.2%). Other colonies belonged to the genera *Pediococcus* (1.85%, 6/325), *Lactobacillus* (0.92%, 3/325), *Streptococcus* (0.62%, 2/325), and *Aerococcus viridans* (0.31%, 1/325). In MSA media, 48 highly resistant colonies were recovered, 60.4% belonging to the genus *Staphylococcus* (29/48) and were represented by 9 different species; *S. epidermidis* n = 7, *S. aureus* n = 5, *S. saprolyticus* (n = 3), *S. haemolyticus* (n = 2), *S. hominis* (n = 3), *S. xylosus* (n = 3), *S. equorum* (n = 2), *S. succinus* (n = 1), *S. pasteuri* (n = 1), and *S. cohnii* (n = 1) and *S. arletae* (n = 1). *Bacillus sp* was identified in 14.6% (7/48) and non-identified species in 29.2% of the isolates (14/48). Details of the species are shown in Table 3.

Intermediate-resistant isolates from M-*Enterococcus* agar seeded with stool samples were identified in 89.1% of the colonial morphotypes tested; most of them (89.1%) corresponded to *Enterococcus*. Within the genus, *E. faecium* was predominant (63.3% of the *Enterococcus*), followed by *E. faecalis* (25.8%), *E. hirae* (3.3%), and *E. durans* (3%). In MSA agar, intermediate-resistant isolates from stool samples accounted for 74.5% of the colonial morphotypes, the majority of them (36.2%) being *Staphylococcus*, mostly *S. epidermidis* (33.3% of them), followed by *S. aureus* (18.5%) and *S. hominis* (16.7%). We recovered an unexpected high number of isolates that corresponded to the genus *Bacillus* (*B. subtilis*, 20; *B. flexus*, 9; *B. licheniformis*, 5; *B. megaterium* and *Bacillus sp*, 4 each; *B. mojavensis* and *B. pumilus*, 3 for each y *B. vallismortis*[1].

Rectal swabs: In the rectal swabs, *high copper-resistant isolates* grown on the M-*Enterococcus* plates (MIC 340 - 512 µg/ml, or 1.36 - 2.05 mM) corresponded to the genera *Enterococcus* (87.1%). As to the proportion of the resistant *Enterococcus species*, 50 isolates out of 81 (61.73%) were identified as *E. faecium*, 25 out of 81 (30.86%) were *E. faecalis* and 6 out of 81 (7.41%) corresponded with two other species (*E. durans* and *E. hirae*). In MSA plates, isolates from 4 different species of high-copper resistance were detected within the genus Staphylococcus (73.3%, 11/15). Details of the species are as follows: *S. aureus* (n = 5), *S. epidermidis* (n = 4), *S. hominis* (n = 1) and *S. warnerii* (n = 1) and 4 of 15 isolates (total of resistant strains) belonged to non-identified bacteria (26.7 %).

At *intermediate copper concentrations* 84% of the resistant isolates obtained in swabs inoculated on M-*Enterococcus* agar plates were *Enterococcus* (89/106) and 4 species were identified, *E. faecium* was the microorganism with the highest

representation (58.4%, 52/89), followed by *E. faecalis* (31.5%, 28/89), and *E. hirae* (5.6% (5/89) of the resistant isolates. Finally, resistant isolates obtained at intermediate concentrations in enriched swabs seeded on MSA agar were mostly *Staphylococcus* (70.8%); within the genus, *S. epidermidis* predominated (47.1%), followed by *S. aureus* (41.2%), *S. hominis* and *S. warnerii* (4.9 % each). Species from *Bacillus* were found in 12.5% of the isolates.

Tables 2 and 3 provide a detailed description of the proportions (per sample and bacterial isolates) of aerobic Firmicutes obtained in M-Enterococcus Agar medium and Manitol-Salt-Agar medium supplemented with copper at high (340 - 512 µg/ml) and intermediate concentrations (170 - 340 µg/ml) of broth-enriched fecal swab samples.

Recovery of copper-resistant bacteria: feces versus rectal swabs

For epidemiological purposes, rectal swabs are much easier to obtain and to handle than feces. Only indirect comparisons of the recovery of copper-resistant bacteria in both types of samples can be made in our study, as each child only provided either feces or swabs. In the case of Gamma-Proteobacteria, culturing broth enriched swabs resulted in a significantly ($p < 0.001$) higher yield of positive samples for high-copper resistant bacteria (65/67, 97%) than direct feces plating (107/166, 64.45%). This difference disappears in recovery for intermediate-resistant bacteria.

The advantage of swabs does not apply for the detection of high- or intermediate copper-R *E. coli*, or *Citrobacter*, and stools are just as effective (not statistically different) as swabs. However, high- or intermediate copper-resistant *Enterobacter* or *Klebsiella* are more efficiently recovered (statistically significant difference, $p < 0.001$) in the swabs. Both fecal samples and rectal swabs yielded a comparable proportion (non-statistically significant) of positive samples for high- or intermediate copper-resistance. The taxon diversity in high- or intermediate-copper resistant species in plates seeded with fecal samples was higher (18 taxa) than that recovered after plating enriched swabs (14 taxa).

Unlike Gamma-Proteobacteria, samples inoculated in M-*Enterococcus* presented no statistical difference in the yield of positive samples for high-level or intermediate-level of copper resistance for the aerobic Firmicutes organisms recovered. As in the case of Proteobacteria, the diversity of Firmicutes with high- or intermediate- copper resistant taxa was higher on plates seeded with feces (13 taxa) than with enriched swabs (5 and 8 taxa, respectively).

Similarly, no statistical difference was detected in the recovery rate of high- or intermediate- copper-resistant strains in fecal or enriched swab samples in MSA agar. As in all former cases, the recovered taxon diversity was higher in the high- or intermediate- copper resistant species in plates seeded with fecal samples (14 and 12 taxa) than that recovered after plating enriched swabs (5 and 7 taxa respectively).

Copper resistance reflects bacterial lifestyle?

Globally we were able to identify 60 different species containing copper-resistant organisms, 22 species of Gamma Proteobacteria and 38 of aerobic Firmicutes (Table 1, and text for *Bacillus* isolates). The genus *Escherichia*, mostly represented by *E. coli*, is constituted by bacterial organisms well adapted to the intestine of the mammals, and is rare in the environment (except in a transient way in sewage, due to fecal contamination). On the contrary, all other Gamma-Proteobacteria detected in this work are able to sustain an environmental lifestyle, that is, are able to reproduce regularly outside animal hosts, frequently with lower optimal growth temperatures than *E. coli*. To a certain extent, some members of the "environmental" genus, as *Klebsiella* (and more rarely *Enterobacter* and *Citrobacter*) are eventually found in association with the vertebrate mucosas, but much less frequently than *E. coli*. We can expect a higher copper exposure in the free environments than in the humans (see Discussion), so that the more adapted intestinal organisms might be "protected" from environmental copper stress. Consistently with these differences in ecology and lifestyle, and using the list of well characterized highly-resistant isolates presented in Table 4, *E. coli* has a significantly lower proportion ($p < 0.001$) of high copper resistant strains (105/211, 49.76%) than the ensemble of environmental Gamma-Proteobacteria (54/70, 77.14%). This difference remains significant comparing E. coli with the ensemble of *Klebsiella, Enterobacter*, and *Citrobacter* (P value of 0.002).

In the case of Firmicutes, ecological and lifestyle differences are less obvious. In the case of *Enterococcus, E. faecalis* is more frequent than *E. faecium* in the human intestine, but both species are able to survive efficiently in the environment. Probably both species are exposed to environmental copper, and *E. faecium* is slightly more copper-resistant (79.27%) than *E. faecalis* (71.28%), but without statistical significance ($p = 0.09$). A similar difference was found comparing *E. faecalis* with all other *Enterococci*. In the case of *Staphylococcus*, the genus has very poor environmental representation; for instance, copper-resistance is not significantly different between the predominant species found, *S. aureus* and *S. epidermidis*.

Proportions and population densities of copper-resistant bacteria in the intestine

A quantitative analysis of the number of colonies obtained in each of the three sectors of the square plates containing low (L), intermediate (M), or high (H) copper concentrations (sector L, < 170 µg/ml or < 0.68 mM, 170-sector M, 170 - 340 µg/ml or 0.68 - 1.36 mM, sector H (340 - 512µg/ml or 1.36 - 2.05 mM) was performed. Percentages of colonies in sector M and H with respect to those in sector L were estimated to offer an estimation of the effect of copper concentration intervals in the survival of bacterial populations, and hence of its possible selective effect. In the case of Gamma-Proteobacteria (Enterobacteriacae), the mean percentages of growth at M and H were, for *E. coli*, 52% and 11%; for *Klebsiella*, 59% and 31%, for *Enterobacter*, 69% and 26%, and for *Citrobacter*, 75% and 32%, respectively. These results show that "environmental Proteobacteria" are not only more frequently copper-resistant than *E. coli*, but also that proportion of the population able to tolerate medium and high copper concentrations is also higher ($p < 0.01$). For Firmicutes, in the case of *E. faecium*, the survivor's frequency in the population in the sectors M and H, compared with the colony count in sector L, was 72% and 39%. For *E. faecalis*, the proportions were 71% and 22% and for *E. hirae*, 57% and 29%, respectively.

The total population size (bacterial counts/ml) of high copper-resistant colonies in fecal samples was reliably estimat-

ed for *E. coli* (103 samples), non-*E. coli* Proteobacteria (57 samples), *Enterococcus* (82 samples) and *Staphylococcus* (28 samples) (Figure 1). The proportion of samples containing ≥ 10^3 cells/ml of sample was higher in *E. coli* (58.25%) than in other Gamma-Proteobacteria (42.10%), which could correspond with the better intestinal adaptation of *E. coli*. This means that, even if *E. coli* is less frequently resistant to copper, the population density of copper resistant *E. coli* in the intestine surpasses other Gamma-Proteobacteria. In the aerobic Firmicutes, copper-resistant *Enterococcus* counted ≥ 10^3 cells/ml in 62.19% of the samples, and *Staphylococcus* in only 14.28%. Considering that *Enterococcus* has high rates of copper-resistance, these results indicate that *Enterococcus* is the main quantitative contributor to high copper resistance in the children's intestine. It should be noted that we were unable to recognize in the distributions presented in Figure 1 any sign of bimodal distribution, meaning that the abundance of copper-resistant bacteria is probably not the result of specific selective events. Lack of bimodality was also obtained in previous studies even using higher copper concentrations (4 - 16 mM)[14].

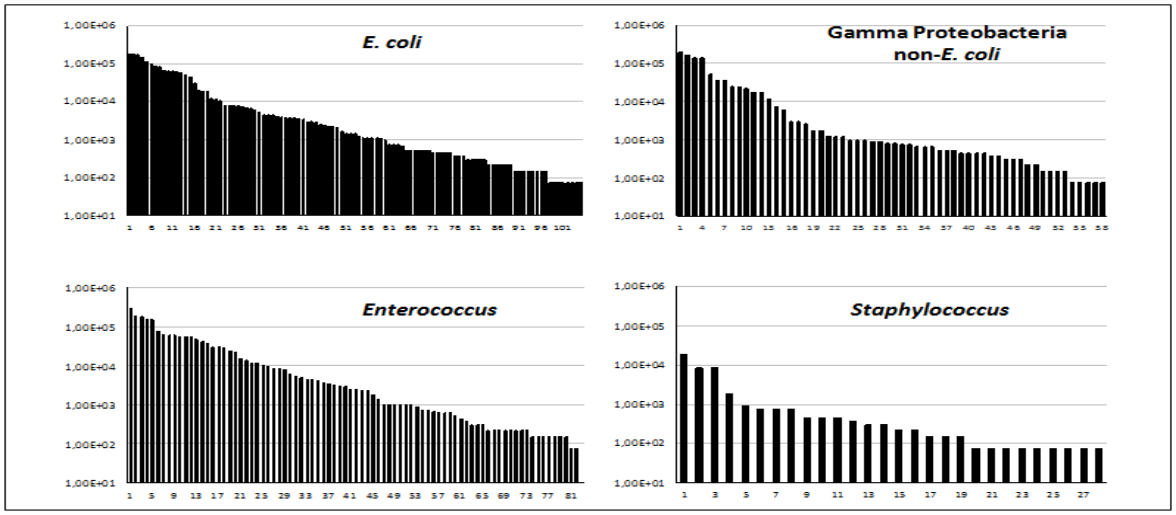

Figure 1: Viable counts (cfu/ml) of different bacterial taxons growing at high copper concentrations (340 - 512 µg/ml).

Children's copper exposure and bacterial copper resistance

A substantial proportion of children's fecal samples contains high copper-resistant (64%) or intermediate-copper resistant Gamma-Proteobacteria (95%). Resistance was significantly higher among organisms with environmental niches than in *Escherichia coli*, suggesting that environmental contamination with copper might be at the origin of copper-resistant bacteria of the children's gut. A high proportion of children's fecal samples (90%) contained copper-resistant Firmicutes, with high predominance of *Enterococcus*, particularly *E. faecium.*

Our group of healthy children had various degrees of copper-concentration in the hair samples, ranging from ≤ 5 to more than 30 mcg/g in dry hair (Figure 2). In order to determine the degree of relationship between the microorganism survival found in a sample and copper levels measured in the sample, the density (colony counts/ml) of the bacteria belonging to the Gamma-Proteobacteria and Firmicutes taxa was plotted against the copper levels measured in the hair sample of the corresponding child, and the Pearson correlation (r) was obtained (Figure 3). In all cases, Pearson r correlation was close to zero ($r = 0.001$ and 0.0002, respectively), indicating the low dependence grade between these variables. Hence, we did not detect any direct (or inverse) relationship between environmental exposure to copper and the microbial counts. As stated in a previous paragraph, copper-resistance is significantly higher in predominantly environmental microorganisms, suggesting that environmental pollution with copper could be the source of copper resistance in bacteria from children's intestine.

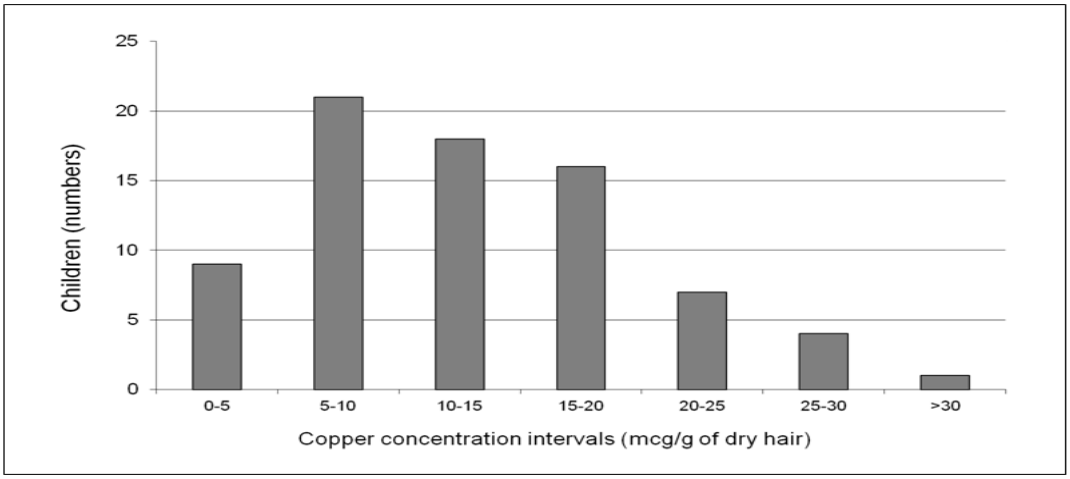

Figure 2: Copper concentrations (mg/g of dry hair) in children.

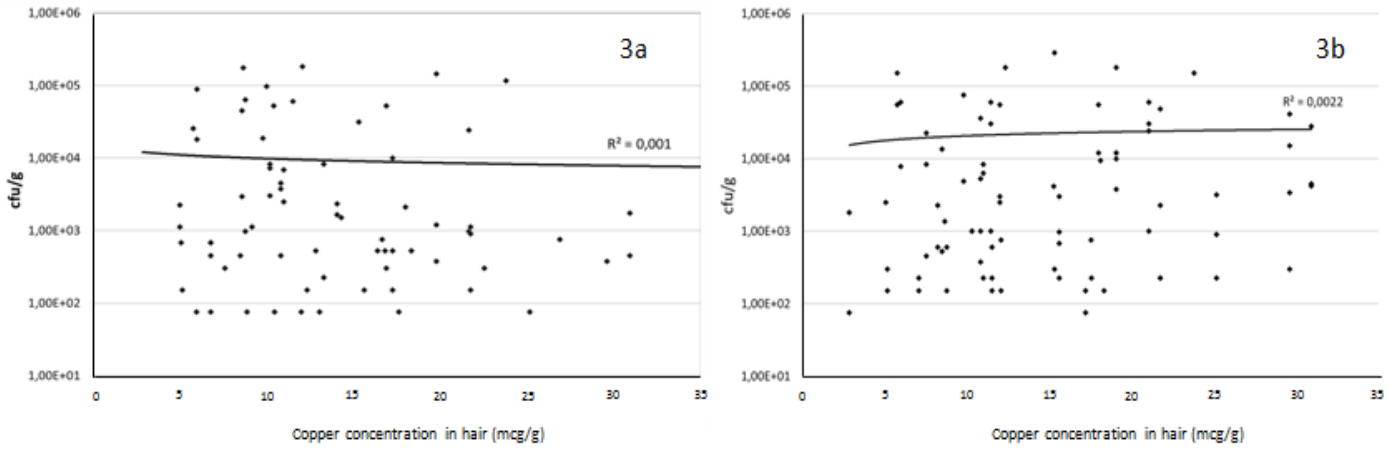

Figure 3: Correlation of copper concentrations (mg/g of dry hear) with bacterial counts of intestinal isolates growing in high copper concentrations (340 - 512 µg/ml). 3a, Gamma-Proteobacteria (recovered in gradient plates of McConkey Agar); 3b, Firmicutes (recovered in gradient plates of M-*Enterococcus* medium)

Discussion

To our knowledge this study provides the most detailed analysis available of copper-resistant microorganisms in intestinal human samples, and particularly in children. Current knowledge about copper-susceptibility of bacterial species from intestinal samples is mainly confined to animals, and based on the screening of Minimal Inhibitory Concentrations (MICs), of a small number of bacterial isolates per sample. Seeding the samples in antimicrobial gradients (as Szybalsky plates) allows evaluation of the effect of toxic agents on natural bacterial populations, particularly when different mechanisms of adaptive tolerance and resistance, that can result in changes in gene expression and a wide phenotypic diversity, can co-exist as in the case of copper. In fact, recent studies show that copper is able to decelerate bacterial growth or even reach lethal concentrations at millimole concentration[16]. Such diversity cannot be captured only by testing individual colonies with conventional MIC methods. Some previous studies focused on particular bacterial groups have used steep gradient plates to detect the phenotype provided by certain genes[17], but never to detect copper-resistance in natural samples, which require a flatter gradient to differentiate small differences in susceptibility.

There are several mechanisms by which bacteria tolerates various levels of copper in their environment. Copper sensing and tolerance might result from relatively unspecific stress response genes[18]. Note that in most cases of copper resistance there is a fuzzy difference between tolerance and resistance, as there are mechanisms of "metalloregulation" in which different genetic mechanisms overlap their phenotypes to protect bacteria against a wide range of toxic (producing reduction of growth rate) copper concentrations, from low to high ones[19,20].

Mechanisms for copper resistance in Gamma Proteobacteria include efflux systems (CusCFBA, CopA-B), copper-binding (CusF, siderophores) and copper-oxidation (peroxidases, CueO). However, many "unspecific mechanisms", such as multidrug efflux transporters (as AcrAB from *E. coli*, or MexB in *P. aeruginosa*), periplasmic chaperones, or proteins involved in the response to superoxydes, act to reduce copper toxicity[21]. Extrachromosomal elements might encode resistance mechanisms to high copper concentrations, frequently regulated by copper-dependent transcriptional regulatory systems (CueR, CusRS, PcoRS)[22]. In the phylum Firmicutes an efflux system, CopA-B, and the CueO gene oxidizing Cu(I) to the less toxic Cu(II), contributes to copper-resistance. A very effective mechanism (involving copper exporting ATPase), elicited by the tcrYAZB operon, assures resistance of exposure to high copper toxic concentrations (>12 mM)[23-26]. Interestingly, CueO and tcrYAZB genes are closely linked, and might be co-regulated by copper sensors CucS, CusR); these genes are often located besides others that also confer resistance to other heavy metals as zinc, arsenic or mercury[24].

We stress that such diversity of mechanisms and phenotypes cannot be reliably captured only by testing individual colonies with conventional MIC methods. Most published studies are focused on copper-resistance in Firmicutes, and a concentration of 12 mM has been considered as the copper-resistant breakpoint of the enterococci[1,2,26]. Of course (see below) the fecal concentrations of copper are probably much lower than the concentration of this "*in vitro*" breakpoint. Most probably many bacterial organisms in a bacterial population that contain genes encoding adaptive mechanisms of copper resistance might be inhibited at much lower copper concentrations (intrapopulation phenotypic diversity). Consistently with this view, sub-inhibitory concentrations of heavy metals have been shown to be sufficient for selecting bacterial populations harboring multi-resistant plasmids with heavy-metal resistances[27]. Concentrations of copper around 1 mM have some effect on Gram-negative sewage bacteria[28]. In fact our study was designed to observe the effect of these relatively low copper concentrations (0 - 2.05 mM).

Which is the copper concentration that might influence the structure of bacterial populations in the intestine? This concentration should depend on the copper uptake. The amount of copper in feed used in growth promotion in pigs[29], frequently exceeds 50 times the amount required for normal growth, and a correlation was found between supplementation of pig feed with such a high copper exposure and the selection of *Enterococci* with specific mechanisms of resistance, as TcrB[30]. However, an increase of 10 fold the recommended concentration for growth

does not affect the MICs of E. coli or *Enterococcus* recovered from feces[25], demonstrating again the difficulty of getting conclusions just from MIC values. It is possible that, as occurs in soil, exposure to low copper concentrations might strengthen the resistance of microorganisms to a subsequent copper stress[31]. If data on dose-response relation for Cu exposure and resistance are lacking, it seems likely that a resistance-driven effect in animals occurs at high trace element exposure rather than at more basal exposure levels[32].

In normal warm-blooded animals ingested copper is absorbed in the stomach and upper small intestine where the maximal concentrations (eventually reaching 10 mM) are expected to occur[19]. In humans, a copper is micronutrient obtained from food and drinking water, which contributes from 0.1 – 1 mg/day, with a tolerated maximum of about 5 mcg/ml[3]; 40% of dietary copper comes from food[3]. Interestingly, one of the key factors of children's increased exposure to copper under natural conditions is the contamination with soil in the environment[33].

Intestinal copper absorption occurs very efficiently, and copper excess is removed by the liver and excretion with bile into feces, in the order of 4.5 mg/day[6]. However, most of the copper secreted with the bile is reabsorbed by enterocytes[34,35], and the copper concentration in Chinese children's blood is around 1 mcg/ml[36]. In addition, the intestinal copper absorption is inversely proportional to the copper content in the diet, being reduced to about one-tenth in high-copper diets[37]. Based on these studies and estimations performed in human samples from autopsies[38], we estimate that the colonic and fecal concentrations of copper would be in the range of 1-2 mcg/ml.

The range of copper concentrations found in dry hair in this study (10 - 30 mcg/g) corresponds to that found in other studies[39]. It is of note that the infants' hair copper levels decline towards the end of the first year of age[40]. Our data on bacterial susceptibility indicate that the expected amount of copper in the children's gut is probably insufficient for selection of bacterial variants with increased copper resistance. In fact, the better adapted Proteobacterial species to the gut, *E. coli*, is significantly less resistant to copper than other Proteobacteria which retain a significant set of functions related with environmental lifestyle. *Klebsiella* organisms contribute to biochemical (as nitrogen fixation) and geochemical earth processes, and have been identified as major components of the microflora in several types of stressed nonclinical environments, including plant surfaces[41,42]. Similar features apply to some of the organisms more frequently interacting with the free environment, as Citrobacter, or *Enterobacter*. In Firmicutes, and in the case of *Enterococcus*, even if *E. faecalis* is more frequent than *E. faecium* in the intestine of humans, both species are able to survive in the environment and be exposed to copper[43].

Our proposal is that most bacteria with increased copper-resistance recovered from the children's gut have evolved in the environment, and not in the human intestine. Probably not in tap water, where the maximum concentration (5 mcg/ml) is about 50 - 100 times lower than the concentrations used in this work to detect copper-resistant bacteria.

The concentration of copper in the environment is obviously variable[44], but concentrations of 10 - 60 mcg/g (roughly/ml) have been found in dry European agricultural soils[45]. Highly efficient resistance mechanisms might have been selected at places where copper concentration exceeds 300 mcg/ml, and these concentration peaks probably occur only in particular environmental areas. In fact, copper was detected in sediments of urban cities (as Stockholm) at a concentration as high as a 475 mcg/ml (Swedish Environmental Protection Agency), similar to the high ones used in our experiments of selection[46]. Usually water-soluble copper compounds occur in the environment after release through activities related with agriculture, mining, smelting, refining and coal-burning industries, and also by natural reasons, as weathering of or the solution of copper minerals[47,48]. Copper is also increasingly used as disinfectant, fungicide or algicide in wood preservation, engraving, lithography or petroleum refining[19]. Moreover, copper discharged to wastewater is concentrated during treatment[3]. In general, the concentration of copper in soil influences the evolution of copper-resistance in the environment at large[49]. Evolved copper-resistant bacteria from the environment might enter in the human intestine.

Our study indicates that among Gamma Proteobacteria *E. coli*, a specialist-intestinal adapted organism, has the lowest proportion of high copper-resistant isolates; however, it is still the most frequent copper-resistant organism in human samples, even in quantitative terms (high population size in the intestine). Other Gamma-Proteobacteria with less intestinal specialization, and harboring traits of environmental lifestyle, seem to have a higher proportion of high-copper resistant isolates, but they are quantitatively much less represented, and their population size in the intestine is lower.

Among aerobic Firmicutes, high-copper resistant *Enterococcus* is very frequently copper-resistant (in particular *E. faecium*), occurs with high prevalence in intestinal samples and has high population sizes, so that its contribution to copper-resistance is the more relevant. Our results are indeed consistent with the observation that intakes of copper were positively associated with the rise of Firmicutes[3]. It should be noticed that the presence of *Staphylococcus* in the stool might be the result of perianal skin contamination, and does not necessarily represent intestinal colonization. It should be finally noticed that copper hair concentrations might not reflect blood concentrations[50], but they allow an easy, non-invasive way of measuring exposure and ingestion influencing intestinal microbiota (not necessarily systemic toxicity).

In summary, we have been unable to document any significant correlation between the copper concentrations in the hair of children and the concentration of copper-resistant Gamma Proteobacteria or Firmicutes in the intestinal samples, and we suggest that the origin of copper-resistance in the children's intestine depends on the populations selected by copper in the external environment.

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