

Bacteriocin Production, Antibiotic Susceptibility and Prevalence of Haemolytic and Gelatinase Activity in Faecal Lactic Acid Bacteria Isolated from Healthy Ethiopian Infants

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Abstract The objective of this study was to characterise lactic acid bacteria (LAB) isolated from faecal samples of healthy Ethiopian infants, with emphasis on bacteriocin production and antibiotic susceptibility. One hundred fifty LAB were obtained from 28 healthy Ethiopian infants. The isolates belonged to *Lactobacillus* (81/150), *Enterococcus* (54/150) and *Streptococcus* (15/150) genera. *Lactobacillus* species were more abundant in the breast-fed infants while *Enterococcus* dominated the mixed-fed population. Bacteriocin-producing LAB species were isolated from eight of the infants. Many different bacteriocins were identified, including one new bacteriocin from *Streptococcus salivarius*, avicin A (class IIa) from *Enterococcus avium*, one class IIa bacteriocin from *Enterococcus faecalis* strains, one unknown bacteriocin from *E. faecalis* and two unknown bacteriocins from *Lactobacillus fermentum* strains and the two-peptide gassericin T from *Lactobacillus gasseri* isolate. Susceptibility tests performed for nine antibiotics suggest that some lactobacilli might have acquired resistance to erythromycin (3 %) and tetracycline (4 %) only. The streptococci were generally antibiotic sensitive except for penicillin, to which they showed intermediate resistance. All enterococci were susceptible to ampicillin while 13 % showed penicillin resistance. Only one *E. faecalis* isolate was vancomycin-resistant. Tetracycline (51 %) and erythromycin (26 %) resistance was prevalent among the enterococci, but multidrug resistance was confined to *E. faecalis* (47 %) and *Enterococcus faecium* (33 %). Screening of enterococcal virulence traits revealed that 2 % were β -

haemolytic. The structural genes of cytolysin were detected in 28 % of the isolates in five enterococcal species, the majority being *E. faecalis* and *Enterococcus raffinosus*. This study shows that bacteriocin production and antibiotic resistance is a common trait of faecal LAB of Ethiopian infants while virulence factors occur at low levels.

Introduction

The gut of a human being is generally considered sterile at birth, but, right from the time of birth, it is colonised by bacteria that increase fast in number and complexity and being established as a durable microflora of the growing infant [1]. The composition of the gut bacteria differs from one to another person [1]. This is because the development and composition of intestinal microflora of infants is determined by various factors [2–4], including feeding type (e.g., breast milk, formula, or other foods), delivery mode (vaginal or caesarean), environment, and hygiene conditions. It has been shown that the colonisation of the gut with lactobacilli and bifidobacteria is delayed in infants delivered by caesarean section compared with vaginally delivered infants [5, 6]. Infants in developing countries are colonised by enterobacteria (including *Escherichia coli*), bifidobacteria, enterococci, and lactobacilli earlier and contain a more diverse microflora than infants from developed countries [3].

The LAB play an important role as probiotics [7–9]. For safety reasons, LAB of healthy human origin, which are non-pathogenic and not resistant to antibiotics, are often preferred for selection as potential probiotics. The probiotic potential of LAB may be promoted by their ability to produce antimicrobial compounds, the most prominent being bacteriocins. Bacteriocins are ribosomally synthesised antimicrobial peptides or proteins produced by bacteria and kill

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other bacteria. Bacteriocin production has also been implicated in the protection of mice against the food-borne pathogen *Listeria monocytogenes* [10]. Thus, bacteriocin-producing LAB can be potential candidates for use as probiotics or as biological control agents.

Even though lactobacilli are generally considered non-pathogenic and clinically less important than enterococci and streptococci, it is necessary to screen them for antibiotic susceptibility. In this regard, EFSA (European Food Safety Authority) has set criteria for safety evaluation of lactobacilli intended for use in animal feeds [11]. This is because there is a concern that lactobacilli (and other normal flora) may act as potential reservoirs of several antibiotic-resistant genes, which might be transferred to pathogenic or opportunistic bacteria during passage in the GIT, thus contributing to the spread of antibiotic resistance [12, 13]. Several studies have shown that the transfer of such genes or plasmids bearing the genes from lactobacilli to pathogens is possible [14–16]. In particular, the *erm(B)* and *tet(M)* genes that confer resistance to erythromycin and tetracycline, respectively, have been identified in intestinal lactobacilli [17].

Enterococci, especially *Enterococcus faecalis* and *Enterococcus faecium*, are the major cause of nosocomial infections [18, 19]. Enterococci display both intrinsic and acquired resistance to antibiotics that entails a challenge to treatment of enterococcal infections. Tolerance to chloramphenicol, erythromycin, tetracycline and high levels of clindamycin, aminoglycosides, β -lactams and glycopeptides is acquired resistance [20, 21]. Acquired resistance is a major concern in infectious diseases not only for posing challenges in the treatment of infections but also for its transmissibility among bacteria. Vancomycin resistance also deserves particular attention since this antibiotic is considered as “a last line of defense” in the treatment of infections caused by multidrug-resistant enterococci [19, 22].

The ability of enterococci to cause diseases can be promoted by production of tissue-damaging virulence factors such as cytolysin and gelatinase [23]. Cytolysin is a two-peptide lantibiotic haemolysin whose production in enterococci is dictated by a cytolysin operon that consists of 8 genes: *cylR1*, *cylR2*, *cylL_L*, *cylL_S*, *cylM*, *cylB*, *cylA* and *cylI* [24]. The operon is mostly located on pheromone-responsive plasmids and sometimes on the chromosome within a pathogenicity island [24]. The occurrence of cytolysin is more prevalent in clinical isolates than in food isolates [25, 26]. Gelatinase production is more frequent in *E. faecalis* and is mainly associated with clinical or veterinary isolates [27, 28].

The objectives of this study were to characterise LAB from faecal samples of infants with emphasis on (1) bacteriocin production, (2) susceptibility to selected antibiotics and (3) detection of cytolysin and gelatinase production, among the enterococci.

Materials and Methods

Subjects and Sample Collection

Faecal samples were collected from 28 healthy Ethiopian infants (Dilla town, Southern Ethiopia), age between 3 and 26 weeks, of whom 16 were females and 12 males. Seventeen were breast-fed, and eleven were mixed-fed. All were vaginally delivered except one. Eight were born at home and 20 in hospital (Table 1). Informed consent of parents was obtained before samples were collected. One faecal sample was collected in sterile screw-capped plastic tubes from each of the infants.

Isolation and Identification of Bacteria

The LAB were isolated on de Man-Rogosa-Sharpe (MRS) supplemented with cysteine–HCl (0.5 gL^{-1}), reinforced clostridial (RC) agar and bile aesculin (BA) agar (all from Oxoid, England). Faecal samples were homogenised in broths (1 g faeces/mL) and tenfold serial dilutions were done. Samples (0.1 mL) were plated from each of the three highest dilutions (10^{-5} to 10^{-7} or 10^{-6} to 10^{-8}) on MRS supplemented with cysteine–HCl (0.5 gL^{-1}), RC and BA agar plates. The plates were incubated under anaerobic condition at $37 \text{ }^\circ\text{C}$ for 24–48 h. Three to four colonies from each plate were picked and inoculated in appropriate stab agar, and transported to Norway. Then, the bacteria in the transport media were streaked on appropriate agar plates to recover the isolates. Single colonies were picked and streaked on plates to confirm their purity. The resulting isolated colonies were subcultured in appropriate broths and subsequently maintained at $-80 \text{ }^\circ\text{C}$ with 13 % glycerol-containing appropriate medium. Species identification was done by partial (about 1,440 bp) 16S rRNA gene sequence analysis which was done by BLAST search against nonredundant database.

DNA isolation, PCR and Sequencing

DNA isolation was done using the Bacterial Genomic DNA Purification Kit (Edge Biosystems, Gaithersburg, USA). The purified DNA was resuspended in $1 \times \text{TE}$ buffer pH 8.

Polymerase chain reaction (PCR) was done on genomic DNA to amplify genes for the 16S rRNA, bacteriocins and cytolysin with appropriate primers (Table 2). The PCR mixture ($25 \mu\text{l}$) contained 100–200 ng template, 0.5 unit DyNAzyme DNA polymerase (Finnzymes), $1 \times \text{DyNAzyme}$ buffer, 0.5 mM MgCl_2 , $200 \mu\text{M dNTPs}$ and $1 \mu\text{M}$ of each primer. PCR conditions—initial denaturation at $95 \text{ }^\circ\text{C}$ for 1 min and 35 cycles at $95 \text{ }^\circ\text{C}$ for 15 s, $50\text{--}54 \text{ }^\circ\text{C}$ for 30 s and $72 \text{ }^\circ\text{C}$ for 90 s. The PCR products were purified using QIAquick PCR purification Kit (QIAGEN, Germany). Cycle

Table 1 Infants characteristics and LAB genera isolated from them

Infant code	Sex	Age (weeks)	Delivery mode	Delivery place	Nutrition	Antibiotic taken	Number of isolates	Number of species	LAB Genera
A	M	5	Vaginal	Hospital	Breast-fed	None	10	3	EL
C	M	4	Vaginal	Home	Breast-fed	None	9	3	L
E	F	11	Vaginal	Hospital	Breast-fed	None	8	3	EL
F	F	14	Vaginal	Hospital	Breast-fed	None	4	3	EL
G	F	9	Vaginal	Hospital	Breast-fed	None	3	2	E
I	M	18	Vaginal	Hospital	Mixed-fed	Unknown	2	2	ES
M	F	16	Vaginal	Hospital	Breast-fed	None	9	3	L
N	M	26	Vaginal	Hospital	Mixed-fed	None	10	4	EL
P	F	7	Vaginal	Hospital	Mixed-fed	None	5	2	L
R	M	8	Vaginal	Home	Breast-fed	None	12	3	L
S	F	18	Vaginal	Home	Mixed-fed	Unknown	10	4	ELS
T	F	26	Vaginal	Hospital	Mixed-fed	Gentamycin	5	1	S
U	F	15	Vaginal	Home	Mixed-fed	None	13	6	ELS
W	F	7	Vaginal	Hospital	Breast-fed	None	5	2	L
X	F	26	Vaginal	Hospital	Mixed-fed	None	7	3	E
Y	F	10	Vaginal	Home	Breast-fed	None	2	2	EL
Z	M	25	Vaginal	Hospital	Mixed-fed	None	4	1	E
3	F	17	Vaginal	Hospital	Breast-fed	None	3	2	EL
5	F	13	Vaginal	Hospital	Breast-fed	Ampicillin	3	2	LS
7	M	11	Vaginal	Hospital	Breast-fed	None	2	2	L
10	M	10	CS	Hospital	Breast-fed	Unknown	4	3	L
11	M	7	Vaginal	Hospital	Breast-fed	None	2	2	EL
12	M	15	Vaginal	Home	Mixed-fed	Cotrimoxazole	4	2	EL
13	M	12	Vaginal	Hospital	Mixed-fed	None	3	2	EL
14	F	14	Vaginal	Hospital	Breast-fed	None	3	1	E
18	F	22	Vaginal	Home	Mixed-fed	None	3	2	EL
20	M	13	Vaginal	Home	Breast-fed	None	1	1	E
21	F	10	Vaginal	Hospital	Breast-fed	None	4	4	ELS

E enterococci, *L* lactobacilli, *S* streptococci

sequencing was done by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), and the PCR products were sequenced by ABI Prism 377 DNA sequencing system (Applied Biosystems, USA).

Bacteriocin Production and Activity

Antimicrobial activity was detected by soft agar overlay assay as described previously [29]. Briefly, the isolated bacteria were spotted onto MRS or GM17 agar plates and incubated for 16 h at 37 °C. The colonies were overlaid with appropriate soft agar seeded with an overnight culture (diluted 400-fold) of the indicator strain. Five indicators (*Lactobacillus sakei* NCDO 2714, *Lactobacillus plantarum* LMGT 2003, *E. faecalis* LMGT 2708, *Listeria innocua* BL86/26 B, *Staphylococcus aureus* LMGT 3242) were used for detecting bacteriocin production (Table 4). After

overnight incubation, the formation of growth-inhibition zones around the colonies was examined as indication of antimicrobial activity. To investigate whether the antimicrobial activity observed was caused by a non-protein compound or a bacteriocin, the antimicrobial activity was tested for sensitivity to proteinase K by adding 1 µl of 20 mgmL⁻¹ proteinase K close to overnight colonies that produce antimicrobial substances. The colonies were then overlaid with appropriate soft agar seeded with an indicator strain and incubated overnight. The next day, the regions close to the colonies where the proteinase K was applied were checked for absence of growth inhibition which is indicative of inactivation of the antimicrobial substance by proteinase K.

In order to further characterise the bacteriocins detected in two *E. faecalis* strains (14M5c and 21M8a), the sensitivity of these strains to other enterococci that produce known

Table 2 DNA primers used in this study

Name	Sequence (5'-3')	Position	Target gene	Reference
1 F	GAGTTTGATCCTGGCTCAG	9–27	16S rRNA	[77]
11 F	TAACACATGCAAGTCGAACG	50–70		[78]
5R	GGTTACCTTGTTACGACTT	1,510–1,492		[79]
<i>gasTF1</i>	GGCTAACATAGTTGGTGGGAGA	2,012–2,033	Gassericin T	[41]
<i>gasTR1</i>	TTTCCGAATCCACCAGTAGC	2,191–2,172		
<i>salAF1</i>	TGATTGCCATGAAAACTCAA	433–453	Salivaricin A	[80]
<i>salAR1</i>	CAACAAACGAATACTGAGTTTGGGA	583–560		
<i>sboAF1</i>	GGCTAAACAACAAATGAATCTCG	19,094–19,116	Salivaricin B	[80]
<i>sboAR1</i>	CAAGTAAACAAGAACTGCCA	19,252–19,233		
BF1	ATGATGAATGCTACTGAAAACCAA	156–179	Bovicin HJ50	[81]
BF2	AATTGTACCTGCACAAATTGAAGA	305–282		
<i>cylLF</i>	CAACAATTTTATGATGGAGGGTAA	42,303–42,326	Cytolysin	[82]
<i>cylSR</i>	TCTTCCATGTAAGCACTCCTTTT	42,831–42,809		
AS-48 F	TTTTTGGGGTTAGCCTTGTT	182–201	AS-48	[83]
AS-48R	GCTGCAGCGAGTAAAGAA	372–355		
Bac31 F	TTTGTGGCATTATTGGGATT	4,276–4,295	Bacteriocin 31	[84]
Bac31 R	CCATGTTGTACCCAACCATT	4,441–4,422		
Ent1071F	ATGCTGTAGGTCCAGCTGC	761–743	Enterocin 1071	[85]
Ent1071R	TTTCCAGGTCTCCACCAGT	552–571		
AVCF2	ACAAGAAAGGCTGTTTCAG	2,010–2,027	Avicin A	[39]
AVCr2	GACTTCCAACCAGCAGCAC	2,109–2,091		

bacteriocins was tested using soft agar assay as described above. *E. faecalis* LMG 2708, which is sensitive to many bacteriocins, including pediocin-like bacteriocins, was used as a reference indicator. The producer enterococci used and their bacteriocins (in bracket) are: *E. faecalis* 100 (enterolysin A), *E. faecalis* 189 (enterolysin A and cytolysin), *E. faecalis* 3207 (enterocin L50 and AS-48), *E. faecalis* 3208 (enterocin P, L50 and AS-48), *E. faecalis* 26c and 116c (enterocin 1071), *E. faecium* L50 (enterocin P, Q, L50) and *E. faecium* T136 (enterocin AB) [30, 31].

Hemolytic Activity

The haemolytic activity of enterococci isolates was tested on brain heart infusion agar supplemented with 1 % (w/v) glucose, 0.03 % (w/v) L-arginine and 5 % (v/v) defibrinated horse blood [32]. The isolates were streaked on the agar, followed by anaerobic incubation at 37 °C for 24 h. *E. faecalis* JH2SS (PAD1) [33] and *E. faecalis* 111A [34] were used as positive controls while *E. faecalis* V583 and *E. faecalis* Symbioflor1 were included as negative controls. Haemolytic activity was detected by the appearance of a clear zone (β -haemolysis), greenish zone (α -haemolysis) or absence of any zone (γ -haemolysis) around the colony on the blood agar. The presence of the cytolysin structural genes (*cylL_L* and *cylL_S*) was verified by PCR and DNA sequencing as described above.

Gelatinase Activity

The ability of the enterococci to produce gelatinase was tested on Todd Hewitt agar (Oxoid, England) supplemented with 3 % bovine gelatine [35]. The bacteria were spotted on the gelatine containing agar plates and incubated at 37 °C for 40 to 48 h, after which they were stored at 4 °C for 5 h. *E. faecalis* V583 was used as a positive control. Gelatinase production was evidenced by the formation a turbid zone around the colonies.

Antibiotic Susceptibility

The sensitivity of the LAB to the following antibiotics (concentration range in micrograms per millilitre) was tested using microtitre plate twofold dilution assay: ampicillin (0.25–512), chloramphenicol (0.5–64), erythromycin (0.125–1024), gentamycin (16–2,048), kanamycin (16–8,192), penicillin G (2–8,192), streptomycin (16–8,192), tetracycline (0.25–32) and vancomycin (0.25–32). Enterococci and streptococci were grown and tested in GM17 and the lactobacilli in MRS. A twofold serial dilution (in broth) of 50 μ l the antibiotic solution was prepared in a microtitre plate well containing 50 μ l broth, to which 150 μ l diluted overnight culture (400-fold diluted in broth) of the test strain was added. The plate was incubated for at least 16 h, after which growth inhibition was measured turbidometrically at

620 nm by a microtitre plate reader (Labsystems Acsent Reader MF, Labsystems, Helsinki, Finland). Resistance was determined by using CLSI (Clinical and Laboratory Standards Institute) breakpoints for enterococci and streptococci and EFSA breakpoints for the lactobacilli [11, 36].

Statistical Analysis

Fisher's exact test was used to compare proportions. *P* value <0.05 was considered statistically significant.

Results

Diversity of the LAB among Infants

A total of 150 LAB were isolated from 28 Ethiopian infants and identified to species level by 16S rRNA gene sequencing analysis. The isolates belonged to *Lactobacillus* (81/150), *Enterococcus* (54/150) and *Streptococcus* (15/150) genera (Table 3). No statistically significant difference in colonisation rates (frequency of occurrence) of lactobacilli was observed between breast-fed and mixed-fed infants (82 % versus 64 %) (Table 3). The same was true for enterococci (59 % versus 82 %) and streptococci (12 % versus 36 %) (Table 3). But the differences could be ecologically significant.

Nevertheless, significant differences were observed in colonisation levels (abundance) of each of the three groups of LAB between breast-fed and mixed-fed babies. Sixty of the *Lactobacillus* isolates (74 %) were found in breast-fed infants which is significantly higher than the number of lactobacilli found among the mixed-fed (21 isolates, 26 %) infants (odds ratio (OR), 5.357; *P*value<0.00001). Conversely, a significant enrichment of enterococci among isolates from mixed-fed infants (59 %) was observed (OR, 2.652; *P*value, 0.0061). The streptococci were more abundant in mixed-fed infants (13 isolates) than in breast-fed infants (two isolates) (OR, 10.057; *P*value, 0.0006) (Table 3).

Bacteriocin Production

The 150 LAB isolates were screened for bacteriocin production using a universal indicator *L. sakei* NCDO 2714 that allows detection of most known class I and II bacteriocins [37–39]. Fourteen strains (Table 4) isolated from eight infants were found to produce antimicrobial compounds that were susceptible to proteolytic treatment. The isolates comprise six *Enterococcus avium*, four *E. faecalis*, two *Lactobacillus fermentum*, one *L. gasseri* and one *Streptococcus salivarius*. From one of the bacteriocinogenic *E. avium* isolates (XA83), a class IIa bacteriocin (avicin A) has

already been purified and characterised [39]. PCR and DNA sequence analysis showed that the other 5 *E. avium* strains (Table 4) from two infants also contain the structural gene of avicin A. The four *E. faecalis* strains (Table 4) produced increased antimicrobial activity up to 6–7 h of growth in liquid GM17 medium at 37 °C, but the activity was gradually lost when incubation time was prolonged. The instability of the bacteriocin produced by *E. faecalis* 21A8a could be due to degradation by gelatinase [40], since this strain produces gelatinase. In order to identify class IIa bacteriocin producers, a sensitive indicator *E. faecalis* LMGT 2708, the resistant mutant *E. faecalis* LMGT 2708RA were used [39]. The faecal isolates *E. faecalis* 14M5a, 14A6b and 14M5c inhibited growth of *E. faecalis* LMGT 2708 while *E. faecalis* LMGT 2708RA was insensitive to the same antibacterial producers. The results strongly suggest that the bacteriocins produced by 14M5a, 14A6b and 14M5c are pediocin-like bacteriocins. PCR and its partial DNA sequence showed that the bacteriocin from *L. gasseri* 10M7c is most likely identical to the two-peptide bacteriocin gassericin T, which inhibits *L. delbrueckii* subsp. *bulgaricus* [41]. *S. salivarius* 5M6c produced a new lantibiotic that is similar to the nisins [42]. PCR did not detect salivaricin A, salivaricin B and bovicin HJ50 in this strain. We have no sequence information of the bacteriocins produced by the remaining LAB isolates (Table 4). Cross-immunity test showed that *E. faecalis* 14M5c and *E. faecalis* 21A8a are sensitive to *E. faecium* L50 and *E. faecium* T136, but they were resistant to *E. faecalis* 3208, 3207, 100, 189, 26c and 116c, suggesting that the bacteriocins produced by strains 14M5c and 21A8a may not be enterocins Q, A or B. PCR was not able to detect genes for AS-48, bacteriocin 31 and enterocin 1071 in strains 14M5c and 21A8a.

β-Haemolytic Activity and Gelatinase Production Are Rare in Gut Enterococci

Fifty-three enterococci were tested for their ability to haemolyse red blood cells. One *E. faecalis* strain (21A8a) was found to be β-haemolytic. The remaining isolates, including the enterococcal negative controls, showed varying degrees of haemolytic activities which were weaker than β-haemolysis. PCR showed that 15/53 enterococci harboured the structural genes for biosynthesis of cytolysin, and the genes were found most frequently in *E. faecalis* and *E. raffinosus* (Table 5).

Gelatinase activity has been observed earlier in faecal enterococci from infants [34]. In this study, two of the 53 tested enterococci (*E. faecalis* and *E. maldoratus*) were found to produce gelatinase. The gelatinase-producing *E. faecalis* (strain 21A8a) showed also bacteriocin and cytolysin activity.

Table 3 Distributions LAB species among infants

LAB species	Number of infants (Number of isolates)			The infants
	Total	Breast-fed	Mixed-fed	
<i>E. avium</i>	6 (16)	3 (5)	3 (11)	G, U, X, 3, 18, 21
<i>Enterococcus canintestini</i>	1 (1)	1 (1)	0 (0)	A
<i>E. faecalis</i>	7 (15)	2 (4)	5 (11)	I, N, S, X, Z, 14, 21
<i>E. faecium</i>	4 (9)	2 (4)	2 (5)	E, S, 12, 20
<i>E. maldoratus</i>	2 (2)	1 (1)	1 (1)	X, Y
<i>E. raffinosus</i>	3 (6)	2 (4)	1 (2)	A, E, U,
<i>E. gallinarum</i>	4 (5)	3 (3)	1 (2)	F, G, 11, 13
Subtotal	19 (54)	10 (22)	9 (32)	
<i>L. fermentum</i>	9 (27)	5 (19)	4 (8)	C, F, M, P, U, W, 10, 12, 18
<i>Lactobacillus mucosae</i>	4 (14)	2 (12)	2 (2)	E, R, S, U
<i>L. casei</i>	4 (12)	2 (8)	2 (4)	A, F, N, P
<i>L. gasseri</i>	6 (9)	5 (7)	1 (2)	C, M, R, U, Y, 10
<i>L. johnsonii</i>	2 (5)	1 (1)	0 (4)	N, R
<i>L. ruminis</i>	1 (4)	1 (4)	0 (0)	W
<i>L. salivarius</i>	6 (8)	5 (7)	1 (1)	3, 5, 10, 11, 13, 21
<i>L. oris</i>	1 (1)	1 (1)	0 (0)	7
<i>L. vaginalis</i>	1 (1)	1 (1)	0 (0)	7
Subtotal	21 (81)	14 (60)	7 (21)	
<i>S. salivarius</i>	1 (1)	1 (1)	0 (0)	5
<i>Streptococcus infantarius</i>	4 (13)	1 (1)	3 (12)	S, T, U, 21
<i>Streptococcus oralis</i>	1 (1)	0 (0)	1 (1)	I
Subtotal	6 (15)	2 (2)	4 (13)	
Total	28 (150)	17 (84)	11 (66)	

Breast-fed infants are shown in bold

Table 4 Bacteriocin production in LAB obtained from faecal samples of Ethiopian infants

Bacteriocin producer ^a	Infant	Indicators (inhibition zone diameter in millimetres)				
		<i>L. plantarum</i> LMGT 2003	<i>L. sakei</i> NCDO 2714	<i>E. faecalis</i> LMGT 2708RA	<i>E. faecalis</i> LMGT 2708	<i>L. innocua</i> BL86/26 B
<i>E. avium</i> XM63	X	0	25	0	15	7
<i>E. avium</i> XA64	X	0	20	0	12	16
<i>E. avium</i> XA83	X	0	25	0	13	8
<i>E. avium</i> XM84	X	0	20	0	11	16
<i>E. avium</i> 3B7b	3	0	25	0	13	15
<i>E. avium</i> 3M7a	3		26	0	12	15
<i>E. faecalis</i> 14M5a	14	0	9	0	6	7
<i>E. faecalis</i> 14M6b	14	0	9	0	7	7
<i>E. faecalis</i> 14M5c	14	0	8	0	7	8
<i>E. faecalis</i> 21A8a	21	0	12	0	0	0
<i>L. fermentum</i> 12M7b	12	10	11	0	0	0
<i>L. fermentum</i> 18M7b	18	0	12	0	0	0
<i>L. gasseri</i> 10M7c	10	0	15	0	0	0
<i>S. salivarius</i> 5M6c	5	0	13	0	0	0

^a All strains were grown in MRS medium except the *E. faecalis* strain that were grown in GM17

Table 5 Haemolytic activity and *cylLS* genes in enterococci

Species	Number of isolates tested	Number of β -haemolysis	Number with <i>cylLS</i> gene
<i>E. avium</i>	16	0	1
<i>E. canintestini</i>	1	0	0
<i>E. faecalis</i>	14	1	6
<i>E. faecium</i>	9	0	2
<i>E. gallinarum</i>	5	0	0
<i>E. maldoratus</i>	2	0	1
<i>E. raffinosus</i>	6	0	5
Total	53	1	15

Antibiotic Resistance Is Common among Faecal LAB from Ethiopian Infants

The antibiotic resistance pattern is different for the three LAB groups (Table 6). The lactobacilli were generally susceptible to chloramphenicol, erythromycin and tetracycline but resistant to aminoglycosides and glycopeptides. Even though many lactobacilli showed sensitivity to ampicillin, the majority of *L. gasseri* did not. Kanamycin resistance was highly prevalent (96 %). One *L. oris*, one *L. vaginalis* and one *L. salivarius* strain were susceptible to kanamycin. Among the *Lactobacillus* species, *L. gasseri* and *L. johnsonii* were sensitive to vancomycin; the rest (83 %) were intrinsically resistant [43].

Most of the *Streptococcus* isolates appeared to be more susceptible to the nine antibiotics than the enterococci (Table 6). All streptococci were susceptible to ampicillin. All streptococci showed intermediate resistance to penicillin (MIC=0.25–2 $\mu\text{g mL}^{-1}$) while all were sensitive to ampicillin, chloramphenicol, erythromycin and vancomycin. One *Streptococcus* isolate was resistant to tetracycline (MIC>8 $\mu\text{g mL}^{-1}$).

E. faecalis and *E. faecium* Constitute a Reservoir for Antibiotic Resistance among the Gut Enterococci

Out of the 53 enterococci isolates tested for antibiotic sensitivity, 34 were resistant to one or more antibiotics. Resistance includes both full resistance and intermediate resistance, unless otherwise stated. Tetracycline resistance was the most prevalent (51 %), followed by erythromycin (26 %) and kanamycin (21 %) and was present in five species. Resistance to ampicillin was not observed. Only one *Enterococcus* isolate showed high vancomycin resistance (MIC>32 $\mu\text{g mL}^{-1}$), and this *E. faecalis* strain was also resistant to penicillin and erythromycin (MIC=32 $\mu\text{g mL}^{-1}$ for both). In addition, two *E. gallinarum* isolates showed intermediate resistance to vancomycin (MIC=8–16 $\mu\text{g mL}^{-1}$).

A high level of aminoglycoside resistance was observed in *E. faecalis* and *E. faecium* isolates. Overall, the prevalence of high level of aminoglycoside resistance was observed in five of fourteen *E. faecalis* and five of nine *E. faecium* isolates. Among these, two *E. faecalis* isolates were resistant to the three aminoglycosides (streptomycin, gentamycin and kanamycin).

Ten isolates (19 %) of the enterococci showed multidrug (more than two antibiotics) resistance, and they included only *E. faecalis* and *E. faecium* isolates. So, multidrug resistance is significantly more prevalent in *E. faecalis* and *E. faecium* than in other enterococci (*P* value, 0.0001). The multidrug resistance observed combined for the most part tetracycline, aminoglycosides and erythromycin resistance.

Discussion

In the present study, 150 faecal LAB from 28 healthy Ethiopian infants were isolated and identified to species level. Their ability to produce bacteriocins and their susceptibility to antibiotics was investigated. Moreover, haemolytic and gelatinase activities were determined. Our study indicates that lactobacilli and enterococci dominate the faecal LAB microflora of Ethiopian infants, consistent with studies of infant gut microflora in other parts of the world [44]. Our results indicate that the lactobacilli are slightly more predominant in the breast-fed infants, but they also constitute a significant part of the faecal LAB flora among the mixed-fed infants. The gut microbiota of the infants is partly acquired during delivery from the vaginal microflora [45], but the mother's milk has also been shown to be an important source of the lactobacilli [46]. A previous study showed that Ethiopian infants contain more enterococci and lactobacilli during the first 2 weeks of life as compared with infants from Sweden [47]. This observation is also in agreement with our result, indicating that this trend extends beyond 2 weeks after birth. Furthermore, two previous studies have shown that enterococci and lactobacilli are

Table 6 MIC range (micrograms per millilitre) and susceptibility of LAB to nine antibiotics

Species (number of strains)	Antibiotic ^a																																		
	Ampicillin			Chloramphenicol			Erythromycin			Gentamycin			Kanamycin			Penicillin G			Streptomycin			Tetracycline			Vancomycin										
	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R	MIC range	S	I	R							
<i>E. avium</i> (16)	0.25–2	16			1–4	16			0.5	16			32	16			32–64	16			2	16			32–128	16			0.5–16	10	5	1	0.5–1	16	
<i>E. canintestini</i> (1)	0.5	1			2	1			16				32	1			256	1			2	1			32	1			0.5	1			1	1	
<i>E. faecalis</i> (14)	0.25–2	14			1–16	12	2		0.5–32	8	5	1	32–512	12	2		32–>2,048	8	6	2–64	12	2		2	32–>2,048	9	5	0.5–>32	7	1	6	1–>32	13	1 ^b	
<i>E. faecium</i> (9)	1–4	9			1–2	9			0.5–8	3	4	2	32–256	9			64–1024	4	5	2–32	5	4		4	64–256	9	0.5–>32	3	3	3	1–2	9			
<i>E. gallinarum</i> (5)	0.5–1	5			1–4	5			0.5	5			32–128	5			128–256	5	2–4	5	2–4	5		5	32–128	5	0.5–>32	3	2	0.5–8	3	2			
<i>E. maldoratus</i> (2)	0.5	2			1–4	2			0.5–1	1	1		32	2			32–64	2		2–128	1			1	32–64	2	0.5	2	1	2					
<i>E. raffinosus</i> (6)	0.25–2	6			4	6			0.5	6			32–64	6			32–256	6		2–8	6			6	128–256	6	>32	6	1–2	6					
Total (53)	53				51	2			39	10	4		51	2			42	42	11	46	7			48	5	26	9	18	>16	50	2	1			
<i>L. fermentum</i> (27)	1–32	22			5	1–2	27		0.25	27			16–128	19	8		128–1,024	8	27	1–32	27			13	32–256	13	14	0.5–2	27			>16		27 ^c	
<i>L. casei</i> (12)	2–8	11			1	0.5–2	12		≤0.25	12			32–64				128–512	12	12	2–4	12			3	64–256	3	9	0.25–0.5	12			>16		12 ^c	
<i>L. gasseri</i> (9)	1–4	4			5	0.5–4	9		≤0.25–128	8	1		16–128	5	4		128–512	4	9	1–4	9			5	16–128	5	4	0.5–2	9		1–2	9			
<i>L. johnsonii</i> (5)	1–2	4			1	1–2	5		≤0.25	5			64–128	5	5		256–512	5	5	1–2	5			2	16–32	2	3	0.5–1	5		1–2	5			
<i>L. mucosae</i> (14)	0.25–4	13			1	1–4	14		≤0.25–0.5	14			16–64	5	9		32–512	9	14	2–16	14			5	32–1,024	5	9	1–2	14		>16			14 ^c	
<i>L. oris</i> (1)	8	1			1	0.25	1		0.125	1			8	1			32	1	4	4	4			8	8	1	0.5	1	>16	1				1 ^c	
<i>L. ruminis</i> (3)	0.25–0.5	3			1	3			0.25	3			16	3			64–512	3	3	1	3			3	16	3	8–16	3	≥16	3				3	
<i>L. salivarius</i> (8)	4–32	6			2	0.5–1	8		0.5–1	8			16–128	4	4		16–2,048	1	7	1–2	7			2	16–512	2	6	0.5–2	8		>16			8 ^c	
<i>L. vaginalis</i> (1)	512	1			1	0.5	1		32				16	1			16	1	8,192	1	8,192			1	256	1	1	>16	1		>16			1 ^c	
Total (80)	63	17			80				78	2	38	42	3	77			4–2,048	3	45	0.25–2	11	8–>1,024	35	45	8	0.125–0.5	11	0.5–1	14			66			
<i>S. infantarius</i> (11)	0.06–0.5	11			0.25–2	11			0.06–0.5	11			4–256				4–2,048	11	11	0.25–2	11			11	8–>1,024	11	0.125–0.5	11	0.5–1	11					
<i>S. oralis</i> (1)	0.0325	1			0.25	1			0.0625	1			32				64	1	1	0.25	1			1	32	1	0.125	1	0.5	1					
<i>S. salivarius</i> (1)	0.0325	1			1	1			0.5	1			32				64	1	1	2	1			1	32	1	0.5	1	0.5	1					
<i>Streptococcus</i> spp (1)	0.125	1			0.5	1			0.0625	1			64				256	1	1	0.5	1			1	128	1	128	1	0.5	1					
Total (14)	14				14				14				14				256	14	14	0.5	1	128	13	14	1	128	1	0.5	1	0.5	1				

S susceptible, I intermediate resistant, R resistant

^a Resistance breakpoints are not available for penicillin for lactobacilli and for glycopeptides for streptococci

^b One vancomycin-resistant isolate has a MIC of 32 µg mL⁻¹

^c Intrinsic resistance

the prevailing LAB in breast-fed Norwegian infants [30, 31], providing more support to our finding. The higher level of occurrence of enterococci in the faeces of mixed-fed than breast-fed infants has previously been attributed to foods other than breast milk [48, 49].

A previous study showed that, in 45 % of Swedish breast-fed infants, lactobacilli reached their highest number at the age of 6 months, the most common being *L. rhamnosus* and *L. gasseri* [50]. *L. rhamnosus*, *L. johnsonii* and *L. paracasei* prevail in the intestinal flora of Greek neonates, but no infants contained more than one species at a time [5]. In our study, these lactobacilli species were also common in the faecal samples of Ethiopian infants; however, the most prevailing species was *L. fermentum*. The most common enterococci isolated from infants are *E. faecalis* and *E. faecium* [3, 51, 52]. Also, *E. avium*, *E. raffinosus* and *S. salivarius* have been identified from neonates [30, 53]. Consistent with these studies, we isolated *E. avium* and *E. faecalis* from many infants and *E. faecium*, *E. raffinosus* and *E. gallinarum* from several samples (Table 3). In this study, the number of infants colonised with enterococci (68 %, Table 3) is higher than what has been reported in a study from Germany in which 23 % of the neonates were colonised with enterococci [52].

Recently, a large number of bacteriocin-producing LAB strains have been isolated from the gut of mammals, including man [54]. Bacteriocin-producing LAB were also found frequently in faecal samples of Norwegian infants [30, 31]. This study also shows that potential bacteriocin producers are commonly found in faecal LAB of Ethiopian infants (10 %) (Table 4). Identification of 14 bacteriocin-like compounds from 150 LAB producers obtained from 28 infants is a relatively high number when compared with isolation of bacteriocin-producing LAB from different environments [55, 56]. Most bacteriocin production was found in enterococci, and this is in line with a similar study of faecal samples from Norwegian infants [30, 31]. It is interesting to note that enterococci which occasionally occur as pathogens are also among the most frequent bacteriocin producers among the gut bacteria of infants [30, 31]. This suggests that enterococci may be better targets (sources) for bacteriocin screening compared with other LAB. In some infants, at least three to six bacteriocin-producing strains belonging to same species were found, suggesting that bacteriocin producers may predominate in their resident gut microflora.

Haemolytic phenotype and genotype have been studied in enterococci obtained from several sources. One study that investigated enterococci from poultry showed that 1 % was β -haemolytic, but 39 % contained the *cylL_S* gene which was most prevalent among the *E. faecalis* isolates [27], and this is similar to the result we obtained. In another study, both cytolysin production and cytolysin operon were detected in 13 % of clinical enterococci [57]. On the contrary,

enterococci isolated from faecal samples of wild boars did not show cytolysin activity or presence of the complete cytolysin operon [58]. Still in another study, β -haemolysis was observed in 33 % and 6 % of clinical and food enterococci, respectively, with high prevalence of *cylL* gene in *E. faecalis* (88 %) and *E. faecium* (70 %) [26]. In *E. faecalis* isolated from healthy Norwegian infants, the prevalence of cytolysin production and cytolysin structural genes was 9/31 and 15/31, respectively [34]. In contrast to the above-mentioned studies, we found very low frequency (2 %) of β -haemolysis activity after 24 h growth on blood plates.

Cytolysin production is common in *E. faecalis* but rare among other enterococcal species. In our study, PCR detected cytolysin structural genes (*cylL_L* and *cylL_S*) also in *E. faecium*, *E. avium*, *E. maldoratus* and *E. raffinosus* (Table 5). Interestingly, one previous study [26], which examined 164 enterococci representing 20 species, detected β -haemolysis under anaerobic incubation in 16 % of the isolates. However, the structural genes of cytolysin were detected only in 9 % of the haemolytic strains, compared with our study which detected the structural genes in 28 % of the enterococci. Cytolysin structural genes were not detected in some haemolytic enterococci. This can be explained by variation in the sequence of the structural genes of cytolysin, or it might be conferred by different haemolysins other than cytolysin, as seen in another study [26]. The detection of structural genes in some non-haemolytic strains (e.g., *E. avium*) might be due to mutations and/or non-functional genes or gene products. It could also be due to loss of some genes from the cytolysin operon.

Gelatinase production is more common in *E. faecalis* [27, 28], although it has been reported in other species such as *E. faecium*, *E. durans* and *E. hirae* [59]. In one study, almost all *E. faecalis* isolated from poultry produced gelatinase [27]. About half of the 31 *E. faecalis* strains isolated from the Norwegian infants produced gelatinase [34]. In the current study, only one out of 15 *E. faecalis* produced gelatinase. These results show that gelatinase production is highly variable among faecal enterococci. In contrast, there are several reports that show that gelatinase production in *E. faecalis* is most prevalent among clinical isolates [28, 60].

Many studies have reported high prevalence of tetracycline resistance among enterococci [61, 62]. This was conferred by acquired resistance mainly due to tetracycline resistant genes *tetL*, *tetM*, *tetS* or *tetO* [61, 63] that is more pronounced in poultry isolates. In one study, it has been shown that tetracycline resistance occurred in 24 % of food isolates [63]. In enterococci isolates from healthy Portuguese children, 29 % of the isolates were resistant to tetracycline, 22 % were resistant to erythromycin, and 9 % were resistant to kanamycin [64]. In another study, a very high level of resistance to tetracycline and erythromycin was present in poultry and pet animals compared with isolates

from faeces of healthy humans [62]. In the same study, no ampicillin resistance was found in human isolates and no vancomycin resistance in any of the isolates [62]. Consistent with these studies, we found high prevalence of tetracycline resistance in the enterococci, followed by erythromycin resistance. Also, tetracycline resistance was the most prevalent (55 %) among *E. faecalis* isolated from healthy Norwegian infants [34], comparable to the present study (53 %). It has been shown in several studies that *E. faecium* is more resistant to penicillin than *E. faecalis* [65, 66]. Our result is in agreement with these studies, since 44 % of the *E. faecium* were resistant to penicillin compared with 13 % of *E. faecalis*. Although we found no enterococci resistant to ampicillin, one *E. faecium* isolate has a MIC value of 4 $\mu\text{g mL}^{-1}$, and according to EFSA criteria, this isolate is not safe for use in animal feeds [67].

Higher prevalence of multidrug resistance (57 %) was reported in enterococci isolated from German neonates [52], as compared with our study (19 %) (P value, 0.0001). The former study also reported absence of vancomycin resistance. Recently, it has been shown that multidrug resistance was frequent in *E. faecalis* clinical isolates [25]. *E. faecalis* was also the most frequent and showed the highest levels of multidrug resistance in the present study. High-level aminoglycoside resistance was also observed among the *E. faecalis* and *E. faecium* isolates, and this might be due to aminoglycoside-modifying enzymes [68]. In contrast to our study, a high level of aminoglycoside resistance occurred in all species of enterococci isolated from poultry but with high prevalence among *E. faecium* and *E. faecalis*.

Many studies have documented the resistance of most lactobacilli, including *L. casei* [69, 70], *L. salivarius*, *L. acidophilus*, *L. plantarum*, *L. delbreuckii*, *L. paracasei* and *L. brevis* [43, 71], to vancomycin which is due to intrinsic resistance. However, some lactobacilli belonging to the *L. acidophilus* group (in our case *L. gasseri* and *L. johnsonii*) were sensitive to vancomycin. This phenotypic characteristic can be used to differentiate the latter group from the rest of lactobacilli [43]. Similarly, the high prevalence of aminoglycoside resistance in the lactobacilli in this study might be due to intrinsic resistance [70]. Eleven *L. casei* strains isolated from Italian hard cheeses were all susceptible to ampicillin, penicillin G, tetracycline and vancomycin, but 1 and 8 were resistant to erythromycin and gentamycin, respectively [72]. In our study, all the 12 *L. casei* were susceptible to tetracycline, chloramphenicol, erythromycin and ampicillin (except one isolate), but all were resistant to vancomycin, gentamycin, kanamycin and most to streptomycin.

A study done on antibiotic susceptibility of intestinal lactobacilli of healthy children showed that all were susceptible to ampicillin, erythromycin and gentamycin and the majority were susceptible to tetracycline while 73 % were

resistant to vancomycin [71]. Except for gentamycin, the study is generally in agreement with our study.

We did not define penicillin-resistant lactobacilli because the relevant breakpoints are not available in either EFSA or CLSI documents, but the MIC values we obtained are generally higher than those found by other studies, especially for *L. fermentum*, *L. gasseri*, *L. salivarius* and *L. vaginalis* [73, 74].

L. casei and *L. johnsonii* in this study were kanamycin-resistant which is in agreement with a previous work of probiotic *L. casei* and *L. johnsonii* isolates that were also shown to be resistant to kanamycin [70]. The same work also showed that the *L. johnsonii* strains were susceptible to chloramphenicol, penicillin and tetracycline, which is also consistent with our study. Also, the high prevalence of kanamycin resistance among the lactobacilli was observed in both studies (81 % in the published probiotic study [70] and 98 % in our study). Lactobacilli are generally more susceptible to chloramphenicol, erythromycin and tetracycline but more resistant to the aminoglycosides [75]. Our result is in agreement with this since nearly all strains were susceptible to chloramphenicol, tetracycline and erythromycin (see Table 4). The only exceptions were the three tetracycline-resistant *L. ruminis* isolates, and the one *L. vaginalis* isolate and one of the *L. gasseri* isolates that were erythromycin-resistant.

It has been shown that nearly 70 % of lactobacilli, including *L. fermentum*, *L. gasseri*, *L. johnsonii* and *L. casei*, isolated from human faeces were found to harbour *tet(M)* and/or *erm(B)* genes [17]. Also, *L. fermentum*, *L. salivarius*, *L. casei* and *L. vaginalis* from food were found to contain these genes, and they were resistant to erythromycin and tetracycline [74]. Therefore, erythromycin resistance observed in our *L. gasseri* and *L. vaginalis* isolates could be due to presence of *erm(B)* genes in these bacteria. In contrast to a study by Delgado et al. [76], we found high prevalence of multidrug resistance in human faecal lactobacilli. However, this multidrug resistance refers to aminoglycosides and vancomycin and should not impose a big concern, since most of these resistances are most likely intrinsic and not transferrable [70, 76].

This work demonstrates that probiotic properties such as bacteriocin production is a major feature among faecal LAB. Some of the isolates shall be evaluated for their potential as probiotics. Also, putative virulence properties like gelatinase and cytolysin activities are rarely found among faecal LAB. The present study also shows that antibiotic resistance is frequently found among most of the faecal LAB.

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