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A Profile Hidden Markov Model to investigate the distribution and frequency of LanB-encoding lantibiotic modification genes in the human microbiome

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Background

The human microbiota plays a key role in health and disease, and bacteriocins, which are small, bacterially produced, antimicrobial peptides, are likely to have an important function in the stability and dynamics of this community. Here we examined the density and distribution of the class I lantibiotic modification protein, LanB, in human oral and stool microbiome datasets using a specially constructed profile Hidden Markov Model (HMM).

Methods

The model was validated by correctly identifying known *lanB* genes in the genomes of known bacteriocin producers more effectively than a model obtained from the Pfam database, while being sensitive enough to differentiate between different classes of lantibiotic modification proteins. This approach was compared with several existing methods to screen both genomic and metagenomic datasets obtained from the Human Microbiome Project (HMP).

Results

Of the methods evaluated, the new profile HMM identified the greatest number of putative LanB proteins in the stool and oral metagenome data while BlastP identified the fewest. In addition, the model identified more LanB proteins than the aforementioned Pfam lanthionine dehydratase model. Searching the gastrointestinal tract subset of the HMP reference genome database with the new HMM identified seven putative class I lantibiotic producers, including two members of the *Coprobacillus* genus.

Conclusions

These findings establish custom profile HMMs as a potentially powerful tool in the search for novel bioactive producers with the power to benefit human health, and reinforce the repertoire of apparent bacteriocin-encoding gene clusters that have been overlooked by culture-dependent mining efforts to date.

Author Cover Page

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Abstract

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Background

Bacteriocins are ribosomally synthesised peptides produced by bacteria that inhibit the growth of other bacteria. Some classes of bacteriocins are post-translationally modified to provide structures beyond those possible by ribosomal translation alone. These modifications are typically key to the peptide's functionality, stability and target recognition (Arnison et al. 2013). Lantibiotics are one such class of small (<5 kDa) modified bacteriocins, possessing characteristic thioester amino acids lanthionine or methyllanthionine (Perez et al. 2014). Lantibiotics form a subgroup within the larger lantipeptide family, which also includes peptides that lack antimicrobial activity. Lantipeptides can be divided into four different classes based on the distinct biosynthetic enzymes responsible for their posttranslational modification (Arnison et al. 2013).

The most commonly studied lantibiotic, Nisin, is a subclass I lantibiotic, meaning that the linear prepeptide is processed by a LanBC modification system (Arnison et al. 2013). Firstly, eight serine and threonine residues in the core peptide are dehydrated by the dehydratase LanB to form dehydroalanine and dehydrobutyrine, respectively (Xie & van der Donk 2004). Secondly, five lanthionine and methyllanthionine crosslinks are formed by the nucleophilic addition of cysteinyl thiols to dehydroalanine and dehydrobutyrine, respectively, by the cyclase LanC (Xie & van der Donk 2004). Finally, the leader sequence, necessary for recognition by the modification enzymes

in the two previous steps, is removed by the protease LanP to produce the active lantibiotic (Xie & van der Donk 2004). The gene-encoded nature of bacteriocins and bacteriocin-like peptides makes them ideal candidates for genome mining. In the case of modified bacteriocins, the structural prepropeptide coding sequence often appears alongside the genes encoding proteins responsible for its modification and export from the cell. However, as more bacteriocins are discovered, the heterogeneous nature of these prepeptides is becoming ever more apparent. This diversity, coupled with their small sequence length, makes bacteriocin prepeptides much more difficult to detect using sequence-homology based searches like BLAST (Altschul et al. 1990). In an effort to address these obstacles, shifting the focus to the detection of bacteriocin-associated proteins opens up more avenues of discovery than simply searching for prepeptide homologs. This provides opportunities to better determine the frequency with which specific types of bacteriocin gene clusters can be found in different environmental niches, such as the human microbiota, through the investigation of metagenomic data.

It has been estimated that the human microbiota comprises approximately 100 trillion bacterial cells, outnumbering our own cells by a factor of 10 or more (Bäckhed et al. 2005). A recent publication, however, has argued that the ratio is actually more likely to be one-to-one, with the numbers being similar enough that each defecation event may alter the ratio to favour human cells over bacteria (Sender et al. 2016). Of greater consequence than bacterial numbers, however, is the collection of genes encoded in this metagenome, thought to be approximately 150 times greater than the human gene complement, with a functional potential far broader than that of its host (Qin et al. 2010). Regardless of absolute numbers, this dynamic community is thought to contain 100-1000 phylotypes (Faith et al. 2013; Qin et al. 2010) and play an integral role in human health and disease (Clemente et al. 2012; Flint et al. 2012). The human microbiota

exhibits robust temporal stability (Belstrøm et al. 2016; Jeffery et al. 2016) perhaps due, in part, to the protection against invading bacteria conferred by bacteriocins and other antimicrobials produced *in situ*. As such, investigation of the density and diversity of bacteriocins produced in the microbiome of healthy individuals may shed light on beneficial and harmful members of this community, and key organisms for maintaining typical i.e. health-associated microbiota composition.

Mining the human microbiota, especially for antimicrobial compounds, has become a popular area of research in recent years (Donia et al. 2014; Walsh et al. 2015). Due to the availability of metagenomic data generated by large public funding initiatives such as the Human Microbiome Project in the U.S. (The Human Microbiome Project Consortium 2012) and the European MetaHIT consortium (Dusko Ehrlich 2010), *in silico* mining of data has emerged as a new tool that has the potential to identify antimicrobial-producing probiotics that can modulate the gut microbiota (Erejuwa et al. 2014; Walsh et al. 2014), or address the increasingly serious threat to public health caused by antimicrobial resistance. There are many available tools for mining the microbiome for antimicrobials, including BAGEL3 (van Heel et al. 2013), antiSMASH (Weber et al. 2015), and traditional sequence-based approaches like BLAST (Altschul et al. 1990). A feature commonly integrated into these tools are Hidden Markov Models (HMM) (Morton et al. 2015; van Heel et al. 2013; Weber et al. 2015) i.e. statistical methods often used to model biological data such as speech recognition, disease interaction and changes in gene expression in cancer (Gales & Young 2007; Seifert et al. 2014; Sherlock et al. 2013). Profile HMMs, a specific subset of HMMs, represent the patterns, motifs and other properties of a multiple sequence alignment by applying a statistical model to estimate the true frequency of a nucleotide or amino acid at a given position in the alignment from its observed frequency (Yoon 2009). Profile

HMMs differ from general HMMs as they move strictly from left to right and do not contain any cycles, a feature that makes them suitable for mimicking the actions of the ribosome during translation. The profile uses three types of hidden states - match states, insert states, and delete states, to describe position-specific residue frequencies, insertions, and deletions, respectively (Yoon 2009). Profile HMMs are potentially more sensitive than sequence homology approaches for identifying more distantly related proteins as they focus on function-dependent conserved motifs that are theoretically slower-evolving, as opposed to focussing on overall sequence similarity. Notably, Skewes-Cox *et al.* successfully designed an approach employing profile HMMs to detect viral protein sequences in metagenomic sequence data (Skewes-Cox et al. 2014).

In this study we designed, validated and implemented a Profile HMM to search for putative subclass I lantibiotic gene clusters in the HMP metagenomes and compared its performance to some of the tools mentioned above.

Methods

Data Collection

HMASM (HMP Illumina WGS Assemblies) and HMRGD (HMP Reference Genomes Data) were downloaded from the Data Analysis and Coordination Centre for the HMP . 835 bacterial RefSeq protein sequences annotated as “lantibiotic dehydratase” were downloaded from NCBI Protein website (13 Apr 2015) in FASTA format.

Building and Validating the new Profile Hidden Markov Model

A multiple sequence alignment was generated in the aligned-FASTA format using MUSCLE (v3.8.31) (Edgar 2004), and a profile HMM was built from the MSA aligned-FASTA file using the HMMER tool hmmbuild (v3.1b1 May 2013) . For comparison of the new model’s

performance, HMMER3's hmmsearch tool was used to search the pfam lantibiotic dehydratase model PF04738 against the same stool and oral HMASM assemblies. Positive and negative controls (listed in Table 1) were used to evaluate the model's ability to 1) accurately identify LanB protein sequences, and 2) distinguish LanB protein sequences from other, related, lantibiotic modification proteins (i.e. LanM and LanL).

Target Sequence Translation

The HMMER3 hmmsearch tool only accepts protein sequences as targets for comparison to protein profile HMMs so a python script was created to translate the nucleotide sequences into protein sequences. The DNA nucleotide sequences were translated in six frames using the standard genetic code.

Metagenomic Screen

The HMMER3 tool hmmsearch was used to search both the new LanB profile HMM and the Pfam PF04738 profile HMM (Punta et al. 2012) against the stool and oral subsets of the Human Microbiome Project's whole metagenomic shotgun sequencing assemblies (HMASM). 139 stool communities and 382 communities from eight different body sites within the oral cavity were screened from the HMP database. These are listed in Table 2. As an additional comparison of performance, a traditional BlastP screen was performed on the same metagenomic samples using the nisin-associated lanthionine dehydratase, NisB, as the driver sequence (GenBank accession number CAA79468.1).

Manual Examination of Randomly Selected Gene Neighbourhoods

A subset of sixty hits were selected and the surrounding region examined to identify other proteins involved in lantibiotic biosynthesis. Open Reading Frames were identified using

Glimmer v3.02 (Delcher et al. 1999), which were then visualised using Artemis (Carver et al. 2012) and blasted against the nr database using BlastP.

Genomic Screen

HMMER3's hmmsearch tool was used to search the new profile HMM against the draft genomes comprising the gastrointestinal tract subset of the Human Microbiome Project's reference genome database.

Results

Validation of the Profile Hidden Markov Model

The ability of the newly developed profile HMM and the pfam lantibiotic dehydratase model PF04738 to detect LanB-encoding genes were compared using the positive and negative controls listed in Table 1. The positive controls selected were all previously characterised bacteriocin producers for which the sequence of the relevant biosynthetic gene cluster was available. A graphical representation of these clusters is presented in Figure 1. *Lactococcus lactis* subsp. *lactis* KF147 was chosen as a negative control because it is of the same subspecies as three of the positive controls (*Lactococcus lactis* subsp. lactis S0, *Lactococcus lactis* subsp. *lactis* CV56 and *Lactococcus lactis* subsp. *lactis* IO-1) but does not produce a bacteriocin. *Streptococcus mutans* GS-5, *Streptomyces cinnamoneus cinnamoneus* DSM 4005 and the *Lactococcus lactis* subsp. *lactis* IL1835 plasmid pES2 were chosen as negative controls to evaluate the ability of the model to differentiate between LanB (subclass I) proteins and the LanM proteins-from these strains, which perform a similar, but distinct, function in the posttranslational modification of class II lantibiotics. *Streptomyces venezuelae* ATCC 10712 was chosen as the final negative control as it has been reported to produce a LanL-type lantipeptide (Goto et al. 2010). Examination of the ATCC 10712 genome using BAGEL3 identified several other orphan lantibiotic modification

genes, including those encoding putative LanL, LanM, LanD and LanB proteins. The genome also appeared to encode a class III lantipeptide cluster comprised of genes potentially encoding a structural protein, two ABC-type transporters and a LanKC modification protein (these genes and clusters are depicted in Figure 2). Notably, there have been no reports of class I lantibiotic production by this strain.

The newly developed LanB profile HMM correctly identified the LanB protein in all nine positive controls, while the PF04738 profile HMM correctly identified the LanB protein in eight of the nine positive controls, failing to detect the Bsa-associated LanB protein in *Staphylococcus aureus* subsp. *aureus* USA300_FPR3757. Both the LanB and PF04738 profile HMMs returned no false positives when searched against the five negative controls used in this study, and, thus, the orphan hypothetical LanB protein reported by BAGEL3 to be encoded in ATCC 10712 genome was correctly regarded as a negative.

Metagenomic Screen

A search with the newly developed profile HMM against the HMASM database identified 399 hits with an E-value of less than $1x10^{-5}$ from the stool metagenomes and 1169 hits with an E-value of less than $1x10^{-5}$ from the oral metagenomes. In contrast, the PF04738 model identified 288 hits with an E-value of less than $1x10^{-5}$ from the stool metagenomes and 686 with an E-value of less than $1x10^{-5}$ from the oral metagenomes. Our model reported at least one putative lantibiotic gene cluster in 81% of oral metagenomes and 86% of stool metagenomes, compared to 73% and 76%, respectively, identified by the Pfam model. The distribution of hits per sample is presented in Figure 3. BlastP identified 231 hits with an E-value of less than $1x10^{-5}$ from the stool metagenomes and 374 hits with an E-value of less than $1x10^{-5}$ from the oral metagenomes. The results of these three approaches were compared to ascertain what proportion of significant

hits was common to more than one search method. The results of this comparison are summarised in Figure 4 and show that the newly developed profile HMM identified the greatest number of lantibiotic modification genes in datasets from both body sites, while the BlastP approach identified the fewest.

The overall results of these combined screening approaches, illustrated in Figure 5 and summarised in Supplemental Table 1, show a higher number and density of hits in the oral metagenomes than in the stool metagenomes and they also reveal a large variation in density of hits between the different sites within the oral metagenomes.

Manual Examination of Selected Gene Neighbourhoods

Sixty hits were randomly selected from those identified by the new profile HMM and manually examined to determine if a bacteriocin gene cluster could be identified. 42% (25/60) of these were not further analyzed because the often relatively short regions assembled from the shotgun data prevented the identification of a full lantibiotic gene cluster. However, of the 35 remaining clusters, 28 (80%) appeared to encode multiple genes involved in the biosynthesis of bacteriocins and thiopeptides. These genes encode proteins involved in posttranslational modification, bacteriocin transport, leader cleavage and regulation (Supplemental Figure 1).

Genomic Screen

The draft genomes of the gastrointestinal tract subset of the HMRGD were also used as a database and searched using the new profile HMM. This resulted in the identification of seven hits with an E-value of less than $1x10^{-5}$, including two strains of *Coprobacillus*, a potentially probiotic genus (Stein et al. 2013; Yan et al. 2012) (Table 3). From these seven genomes, only three lantibiotic gene clusters were identified by BAGEL3, these are illustrated in Figure 6. Although this low frequency of lanthionine dehydratase proteins in the dataset contrasts with the

findings of the metagenome screen reported above, it is in agreement with previous reports of relatively low class I lantibiotic density within the human microbiota (Walsh et al. 2015; Zheng et al. 2014). A possible explanation for this is that the class I lantibiotic clusters identified in the metagenomics data by the new profile HMM are present in the genomes of rarer members of the gut microbiota, which are not represented in the HMP reference genome database.

Discussion

Bacteriocin production enhances the competitiveness of bacteria living in complex communities and has the potential to be harnessed for the benefit of human health. The goal of this study was to develop a profile HMM and to assess its ability, in comparison with several other approaches, to detect putative subclass I lantibiotic gene clusters in human metagenomic datasets. Through this process, it was also possible to evaluate the potential density and distribution of these bacteriocin gene clusters in the human microbiota.

To validate the model, nine positive controls and five negative controls were selected to evaluate its sensitivity and specificity. These controls were selected based on reported bacteriocin production; the positive controls were all known producers of class I lantibiotics while the negative controls produced either different classes of lantibiotics or none at all. Following validation, genomic and metagenomic data corresponding to two niches within the human microbiome were chosen as the focus of this study. The first of these niches was human stool and was selected as the corresponding samples were most likely to yield bacteriocin producers with the potential to modulate undesirable microbiota profiles associated with obesity, colorectal cancer, type 2 diabetes or inflammatory bowel diseases due to their ability to survive and colonise this environment. Secondly, human oral communities were examined as a previous study by Zheng *et al.* showed that they contained, by far, the greatest percentage of bacteriocin

structural genes across a number of human metagenome samples (Zheng et al. 2014). Zheng *et al.* reported that 80% of class I bacteriocins (lantibiotics) and 89% of all bacteriocins identified using their method originated in the oral metagenomes, while the stool metagenomes contained just 15% and 7%, respectively. The same study reported that 88% of samples from the oral cavity and 73% of samples from the gut contained at least one bacteriocin (regardless of class), while the new profile HMM reported these statistics as 81% and 83%, respectively for sub- I lantibiotics alone. The *in silico* screen carried out with the profile HMM is consistent with the observation by Zheng *et al* (Zheng et al. 2014) by yielding a higher number and density of hits from the oral, compared to the stool, metagenomic data. Furthermore, the large variation in density of hits between sites within the oral environment suggests that lantibiotic production confers a greater advantage in subgingival plaque, supragingival plaque, and tongue dorsum communities compared to communities from the throat and buccal mucosa. This may be due to the direct benefits of antimicrobial activity but could also involve the intra- and interspecies signalling roles attributed to lantibiotic peptides (Upton et al. 2001).

One of the most interesting observations from the study was the large variation in the numbers of *lanB* genes reported by the three different approaches. The BlastP approach identified, by far, the lowest number of significant hits overall and the lowest in every body site examined, except for the saliva microbiome. Our model identified more than double the number of hits provided by the BlastP-based approach. This is to be expected as profile HMMs are known to typically outperform pairwise sequence comparison methods (such as BLAST) in the detection of distant homologs (Park et al. 1998). Our model also identified a greater number of LanB proteins than the Pfam PF04738 model when used to search the same data using the same parameters. While the PF04738 model relates to the N-terminus of the lanthionine dehydratase protein, responsible

for the serine-threonine glutamylation step of lantibiotic modification (Ortega et al. 2015), the newly developed profile HMM takes the full length of the LanB protein into consideration, thereby providing greater predictive power.

Zheng *et al*., using the same metagenomic data that was the focus of this study, identified 17 class I lantibiotics from stool samples and 76 from oral samples, a much lower frequency of detection than in this study, probably due to the different methodologies used. That study focused on searching for proteins similar to those in BAGEL3's manually curated database, an approach which likely lost sensitivity because bacteriocin precursor peptides can differ considerably at primary sequence level. Furthermore, the screen employed a BLAST-based approach which, as demonstrated here, exhibited the lowest number of significant hits reported.

To investigate the areas surrounding the LanB-encoding genes identified by our model we randomly selected thirty positive hits from the oral and stool metagenome screens for manual examination. This approach revealed that several of the hits were on scaffolds that were either too small to contain a full gene or did not contain the gene's start codon. This was most likely as a consequence of the fragmented nature of the metagenomic data, as opposed the identification of true false positives by the model and would probably occur regardless of the method employed. 42% (25/60) of hits selected for manual examination were discarded based on these criteria. It also revealed that a considerable number of hits exhibited low (~30%) similarity to putative thioesterases in the nr protein sequence database, highlighting that lanthionine dehydratases are relatively-closely related to proteins involved in the posttranslational modification of thiopeptides, most likely those responsible for dehydration of serine and threonine residues (Garg et al. 2013). The similarity between these dehydratase proteins suggests a possible common ancestor protein (Kelly et al. 2009). Another possible explanation relates to

the fact that all of the proteins annotated as thiopeptide modification proteins are putative annotations and none, to our knowledge, have been confirmed as such *in vitro*. It is possible, therefore, that these may simply be lanthionine dehydratases which have been incorrectly annotated due to automatic software and incomplete/under-curated databases. The majority of clusters identified contained genes encoding both LanB and LanC modification proteins as well as a leader cleavage and activation peptidase, and ABC transporter proteins for export of the mature peptide, suggesting that these have the potential to encode a functional lantibiotic.

To evaluate the model's performance in a genomic context we applied it to the gastrointestinal tract subset of the HMP's reference genome database and compared the results to our previously published study which used the online bacteriocin genome mining tool BAGEL3 (van Heel et al. 2013) to screen this same database (Walsh et al. 2015). The results of the two screens were startlingly different and served to highlight the variation in results that can arise from applying different methods to the same data.

Conclusions

Across the oral and stool communities examined, this study identified 2007 unique putative subclass I lantibiotic biosynthetic gene clusters, further emphasising the tremendous potential that the human microbiota has as a source of therapeutic compounds. The next challenge lies in correctly identifying those elements with the ability to desirably modulate the microbiota and utilizing them in the treatment of microbiota-associated disease.

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List of Abbreviations

Abbreviation	Description
HMASM	Human Microbiome Project's Illumina Whole Genome Shotgun Assemblies
HMM	Hidden Markov Model
HMP	Human Microbiome Project
HMRGD	Human Microbiome Project's Reference Genome Data

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Figure 1

BAGEL3 output of putative bacteriocin gene clusters identified in positive controls used in validation of our new profile HMM.

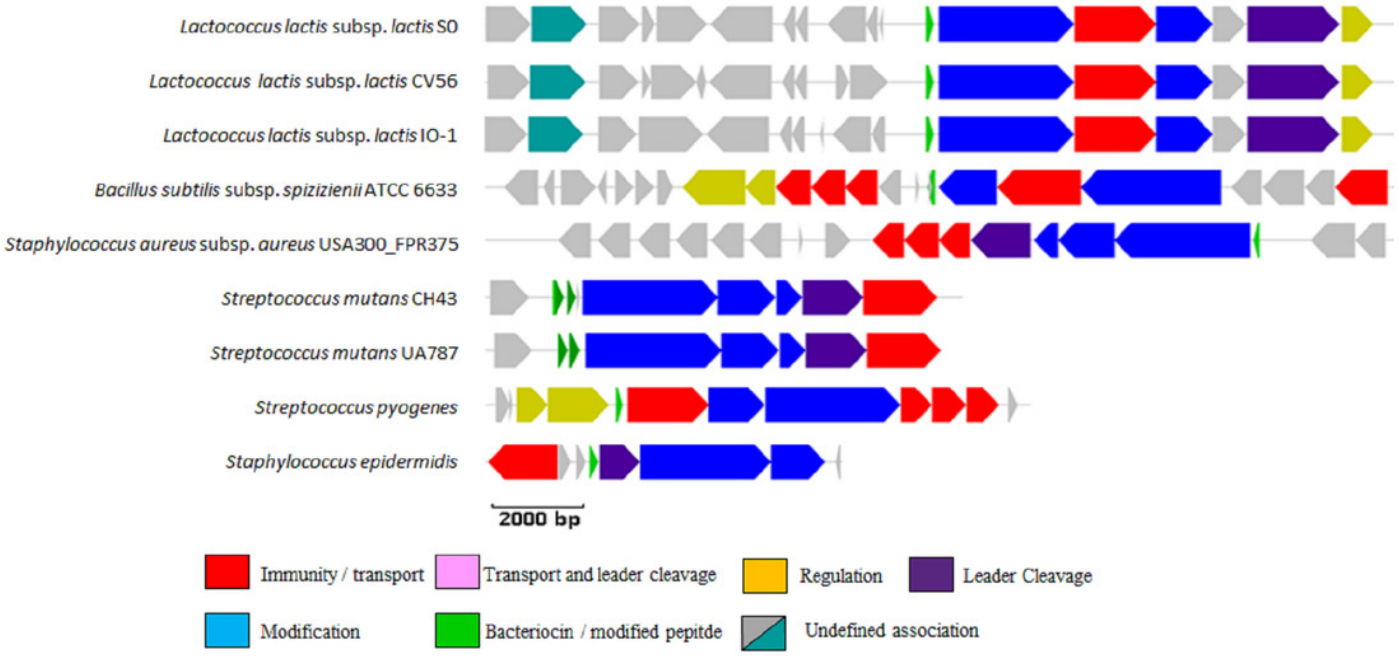


Figure 2

BAGEL3 output of putative bacteriocin gene clusters identified in negative controls used in validation of our new profile HMM.

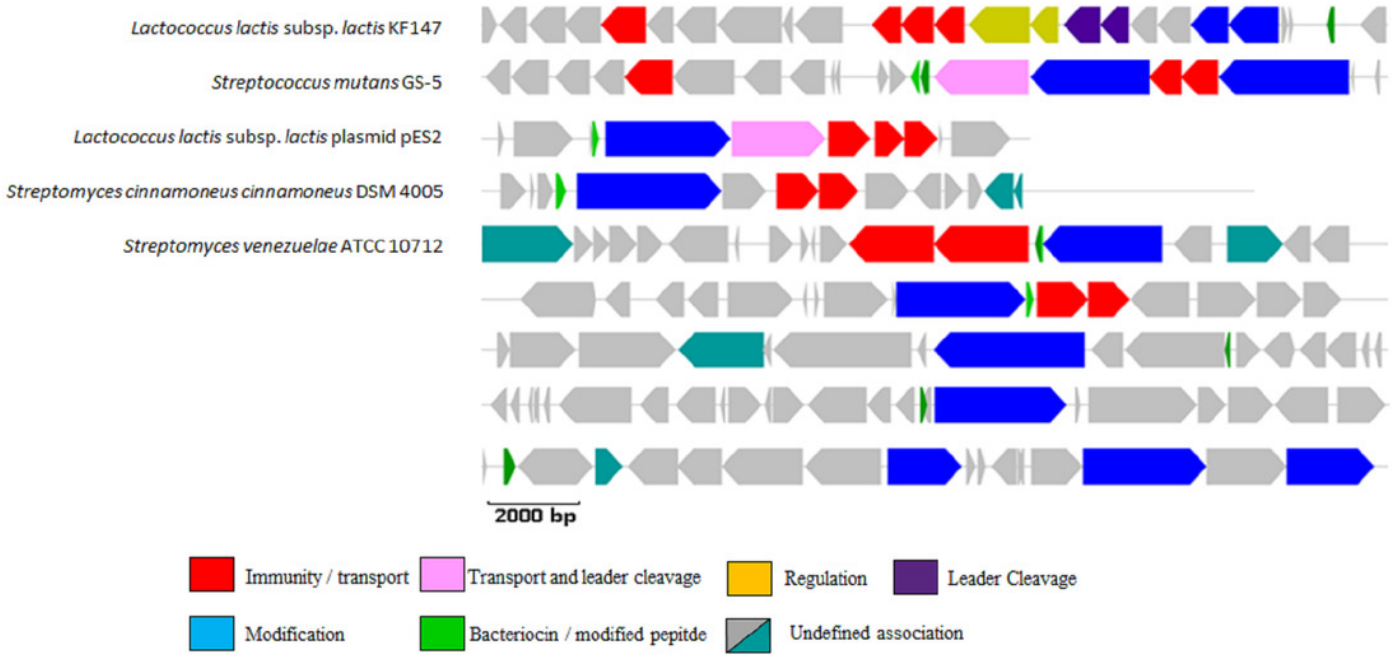


Figure 3

Distributions of lanthionine dehydratase proteins per sample identified by our new profile HMM.

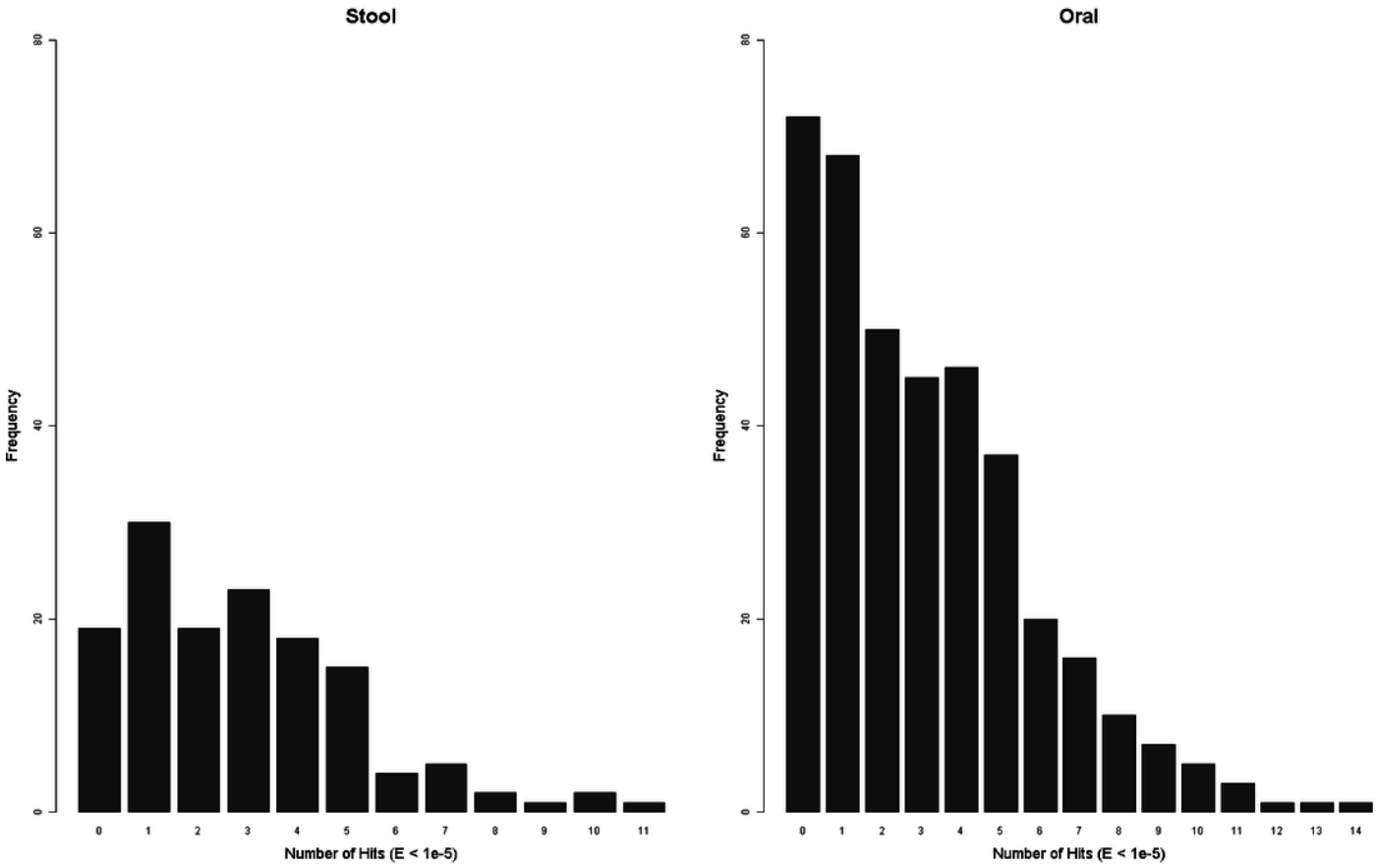
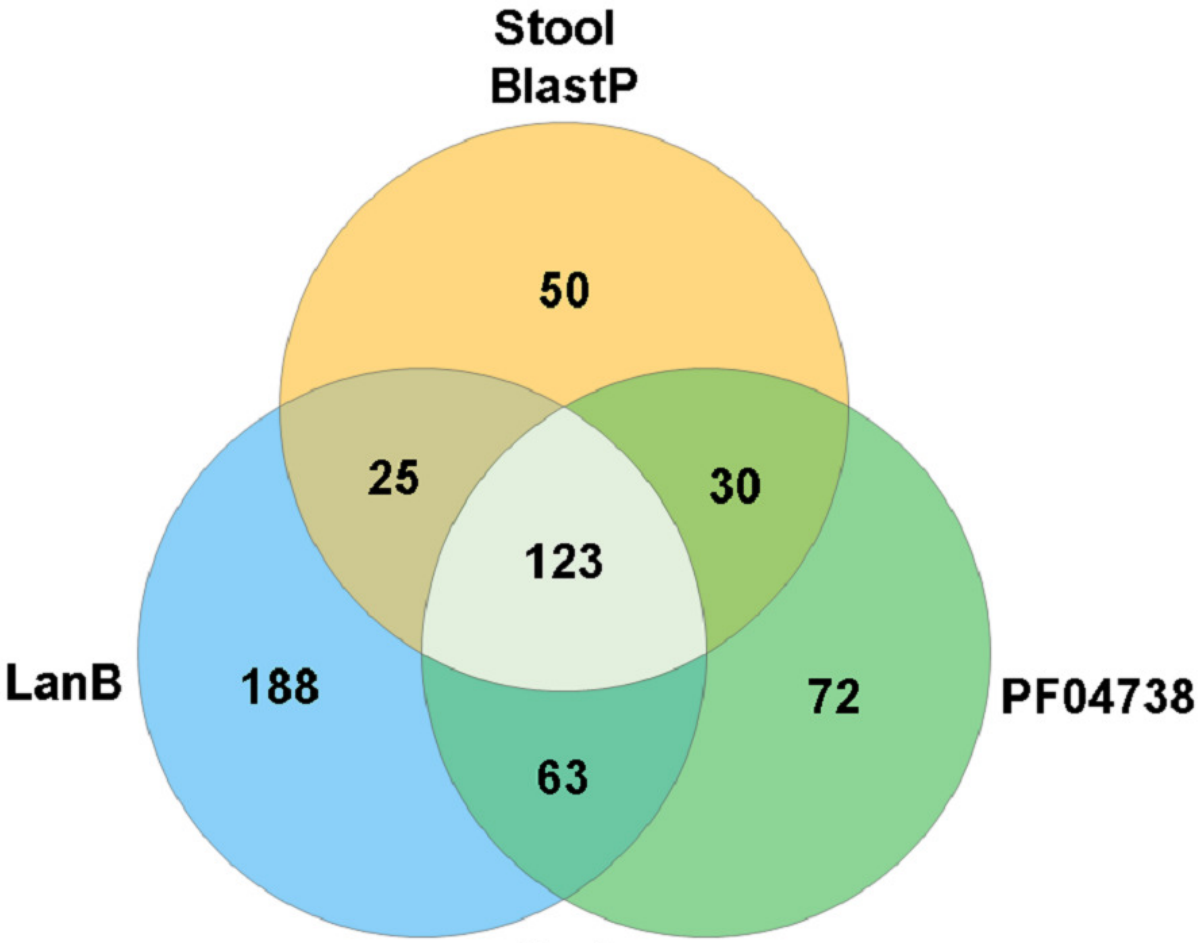


Figure 4

Numbers of lanthionine dehydratase proteins reported by single and multiple methods.


Stool
BlastP
50
25
30
123
LanB
188
72
PF04738
63

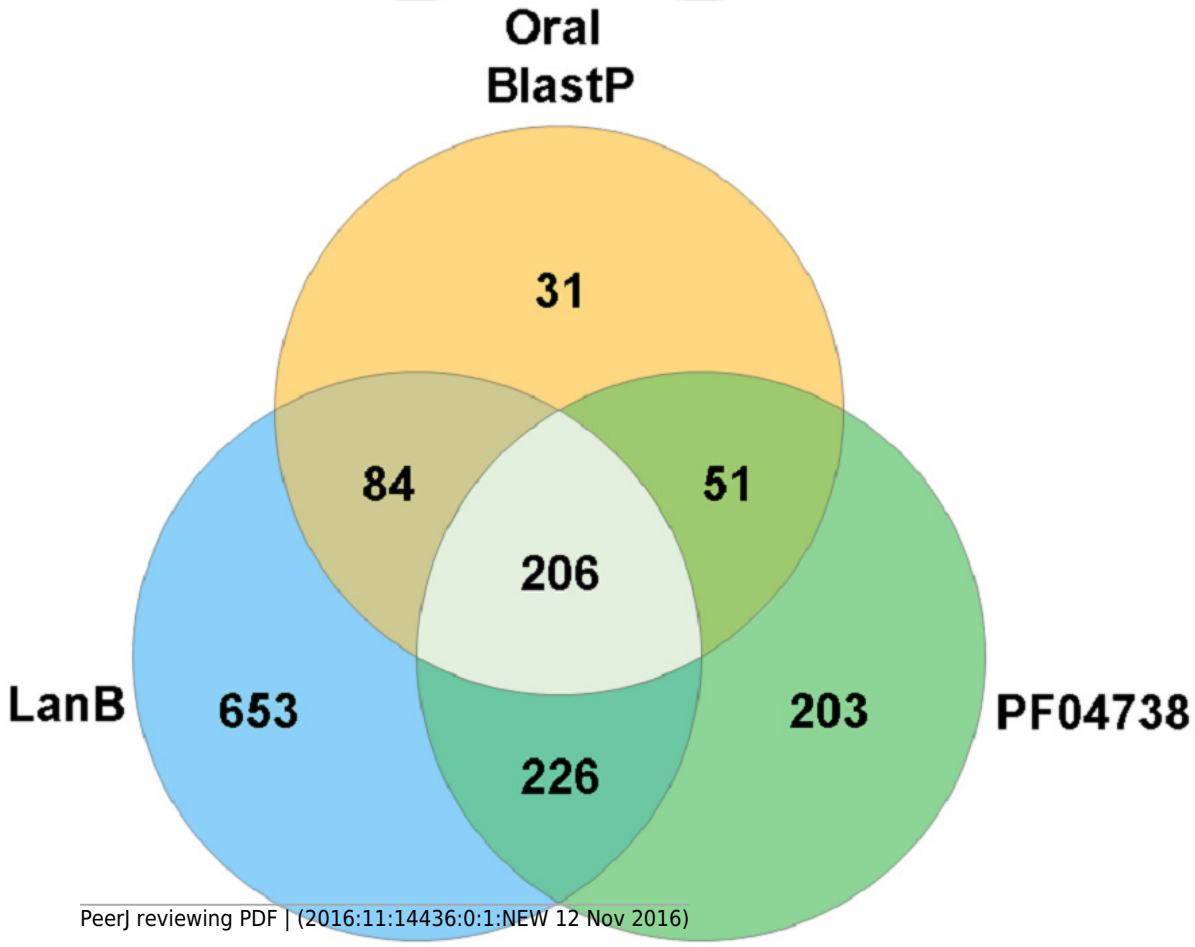

Oral
BlastP
31
84
51
206
LanB
653
203
PF04738
226

Figure 5

Comparison of lanthionine dehydratase density by body site reported by all three methods. Insert shows overall comparison between stool and oral environments.

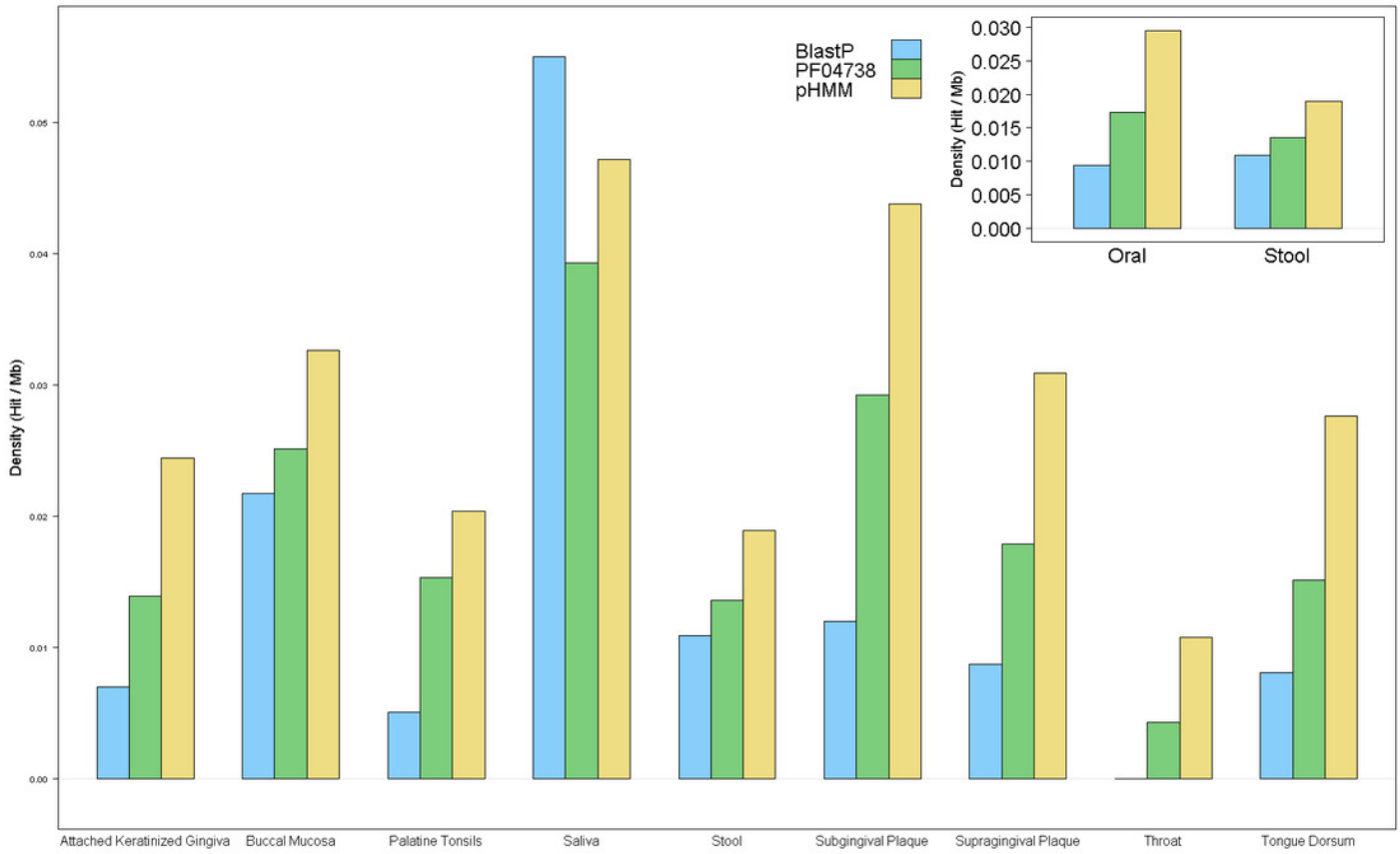


Figure 6

BAGEL3 output of three putative bacteriocin gene clusters identified from the gastrointestinal tract subset of the Human Microbiome Project's reference genome database by our new profile HMM.

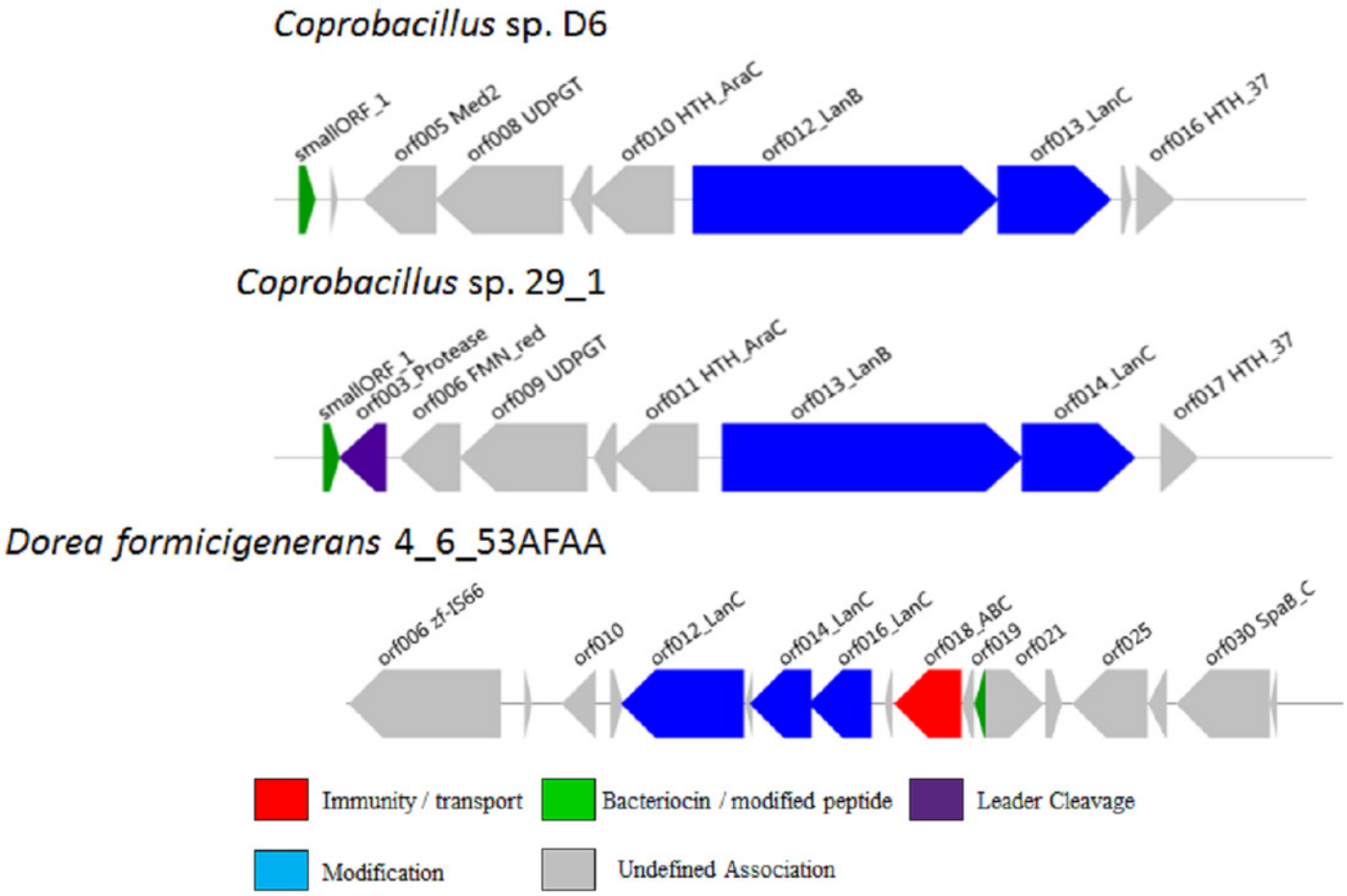


Table 1 (on next page)

Controls used in validation of the profile HMM.

[a] Relevant lanthionine dehydratase protein was correctly identified by our model

[b] Relevant lanthionine dehydratase protein was correctly identified by PF04738

[c] No lanthionine dehydratase protein identified by either model

Strain	Bacteriocin	Class
Lactococcus lactis ssp. *lactis* S0[a,b]	Nisin Z	LanB
Lactococcus lactis ssp. *lactis* CV56 [a,b]	Nisin A	LanB
Lactococcus lactis ssp. *lactis* IO-1 [a,b]	Nisin Z	LanB
Bacillus subtilis subsp. *spizizienii* ATCC 6633 [a,b]	Subtilin	LanB
Staphylococcus aureus subsp. *aureus* USA300_FPR3757 [a]	Bsa	LanB
Streptococcus mutans CH43 [a,b]	Mutacin I	LanB
Streptococcus mutans UA787 [a,b]	Mutacin III	LanB
Streptococcus pyogenes [a,b]	Streptin	LanB
Staphylococcus epidermidis [a,b]	Pep5	LanB
Lactococcus lactis subsp. *lactis* KF147 [c]	None	-
Streptococcus mutans GS-5 [c]	Mutacin GS-5	LanM
Lactococcus lactis subsp. *lactis* plasmid pES2 [c]	Lacticin 481	LanM
Streptomyces cinnamoneus cinnamoneus DSM 4005 [c]	Cinnamycin	LanM
Streptomyces venezuelae ATCC 10712 [c]	Venezuelin	LanL

1

Table 2 (on next page)

Samples per body site screened.

Site	Number of Samples
Attached Keratinized Gingiva	6
Buccal Mucosa	107
Palatine Tonsils	6
Saliva	3
Stool	139
Subgingival Plaque	7
Supragingival Plaque	118
Throat	7
Tongue Dorsum	128

Table 3 (on next page)

Lanthionine dehydratase proteins identified in the gastrointestinal tract subset of the Human Microbiome Project's reference genome database using our profile HMM.

Accession	Strain	E Value
JH414709	*Bacillus* sp. 7_6_55CFAA_CT2	9.0E-16
GL636578	*Coprobacillus* sp. 29_1	3.7E-67
AKCB01000002	*Coprobacillus* sp. D6	4.5E-68
JH126516	*Dorea formicigenerans* 4_6_53AFAA	2.3E-81
ACEP01000029	*Eubacterium hallii* DSM3353	9.4E-27
KI391961	*Fusobacterium nucleatum* subsp. *animalis* 3_1_33	2.2E-09
GG657999	*Fusobacterium* sp. 4_1_13	7.1E-09