



Resistance of zebu cattle (*Bos indicus*) to colonization by major ruminant hoof pathogens

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ABSTRACT

Zebu cattle (*Bos indicus*) is reported to be more resistant towards harmful environmental factors than taurine cattle (*Bos taurus*). A few hundred zebu cattle are kept in Switzerland and in contrast to the Swiss indigenous breeds, infectious hoof disease in zebu is not observed. Therefore, we compared the prevalence of three ruminant hoof pathogens in zebu and taurine cattle. These included *Treponema* spp., *Fusobacterium necrophorum* and *Dichelobacter nodosus* which are associated with bovine digital dermatitis (BDD), different bovine hoof diseases and ovine footrot, respectively. Interdigital swabs and punch biopsies from hind feet of slaughter animals were tested for the three pathogens by PCR. Sixty zebu from eight farms were compared to a convenience sample of 20 taurine cattle from 17 farms. *Treponema* spp. associated with BDD were not detected in zebu while 23 % of animals and 50 % of farms were positive for benign *D. nodosus*, with results indicating environmental contamination rather than colonization. Taurine cattle showed 35 % of animals and 41 % of farms positive for *T. phagedenis* while 90 % of animals and 94 % of farms were colonized by *D. nodosus* as indicated by a 500-fold higher bacterial load than in zebu. The difference in prevalence of the two pathogens between zebu and taurine cattle was highly significant. *F. necrophorum* was as well only detected in taurine cattle with values of 15 % of animals and 17.7 % of farms, being significantly different at the animal level. Furthermore, genetic analysis of Swiss zebu indicates high genomic diversity and clear separation from taurine cattle. This is the first evidence that zebu show resistance towards colonization by bacterial hoof pathogens in contrast to taurine cattle.

1. Introduction

About 300,000 years ago humped *Bos indicus* (zebu cattle) and hump less *Bos taurus* (taurine cattle) populations diverged from their extinct aurochs progenitor *Bos primigenus* (Utsunomiya et al., 2019). Domestication of cattle then started about 10,000 years ago with indicine cattle having their origin in India, while taurine cattle in the Near East (Bonfiglio et al., 2012; Edwards et al., 2007; Utsunomiya et al., 2019). Indicine cattle such as zebu have a distinct morphology with fatty hump on their shoulders, large ears and a dewlap. While a higher resistance of zebus towards heat, poor nutrition and a number of parasites compared to taurine cattle is well known (Bock et al., 1997; Glass et al., 2005; Utsunomiya et al., 2019; Wambura et al., 1998) differences in colonization by different bacterial pathogens are poorly investigated. Zebu were introduced to Switzerland about 30 years ago and kept in suckler cow husbandry. Talking to zebu breeders revealed that generally they have no problems concerning hoof diseases in contrast to taurine cattle

farms. In a recent study more than 75 % of taurine cows in Switzerland showed claw problems and the prevalence of bovine digital dermatitis (BDD) at single animal level was more than 20 % (Jury et al., 2021). BDD, also known as Mortellaro, is a multifactorial disease that different treponemes are associated with, in particular *Treponema phagedenis*, *Treponema pedis* and *Treponema medium* (Sullivan et al., 2013). Besides *Treponema* spp. other bacteria like *Dichelobacter nodosus* or *Fusobacterium necrophorum* are supposed to be present in BDD (Rasmussen et al., 2012; Sullivan et al., 2015). Studies on taurine cattle as a potential reservoir for *D. nodosus*, the etiologic agent of ovine footrot, revealed a high prevalence of benign *D. nodosus* (aprB2-positive) in clinically healthy feet of more than 80 % while virulent *D. nodosus* (aprV2--positive) was so far not detected in Swiss taurine cattle (Alsaad et al., 2019; Arduser et al., 2020). The role of *D. nodosus* in bovine hoof diseases is not clear. Similarly, the role of *F. necrophorum* remains vague since it can also be found in healthy feet (Sullivan et al., 2015). Nevertheless, it is associated with necrobacillosis in taurine cattle which

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in the feet manifests itself as interdigital phlegmon also referred to as bovine footrot (Van Metre, 2017). To investigate the prevalence of the mentioned pathogens in zebu and taurine cattle, we sampled corresponding feet from slaughtered animals and screened for the presence of different bacteria by PCR. In parallel the genetic background of Swiss zebu cattle was assessed.

2. Material and methods

2.1. Selection of farms and collection of feet

Eight zebu farms located in 4 different Swiss cantons were included in the study (Table 1). One of them (farm D) raised crossbreed (*Bos indicus* x *Bos taurus*) and the other seven purebred zebu (*Bos indicus*). Both hind legs from 60 recently slaughtered animals were collected at five different slaughterhouses between November 2021 and April 2023. At least 2 animals per farm were tested. Convenience samples of hind feet from 20 taurine cattle (*Bos taurus*) originating from 17 different farms were collected at two slaughterhouses in January and February 2023 (Table 1). Feet collected at slaughter were transported to the laboratory at ambient temperature and subsequently processed within 2–16 hours.

2.2. Specimen collection and DNA isolation

Claws were cleaned from gross dirt and visually inspected for lesions. Cotton swabs, useful for detection of *D. nodosus* (Arduser et al., 2020) and tissue biopsies, routinely used for detection of BDD *Treponema* spp. (Sullivan et al., 2015) were then taken. The interdigital cleft and heel bulb from feet of 55 zebu and the 20 taurine cattle were rubbed with a cotton swab. The swab was then placed in a microtube containing 1 ml of lysis buffer (4 M guanidine thiocyanate, 0.01 M Tris- HCl, 1 % beta-mercaptoethanol) for 1 min and then squeezed and discarded. Additionally punch biopsies (6 mm; Kai medical, Japan) were taken from feet of 49 zebu and the 20 taurine cattle from the heel bulb where normally BDD lesions are observed (Caddey et al., 2021; Plummer and Krull, 2017; Thomas et al., 2022; Vanhoudt et al., 2023). The biopsy was sliced into smaller pieces with a scalpel in a sterile petri dish and then placed in a microtube containing 1 ml lysis buffer. The swab and biopsy containing microtubes were stored over night at 4°C. DNA extraction was performed from 500 µl lysate by a semi-automated extraction robot (KingFisher™ DuoPrime, Thermo Fisher Scientific, Reinach, Switzerland) using magnetic beads (Stauble et al., 2014b). Prior to starting extraction, the Internal Positive Control (IPC) DNA (20,000 copies; Thermo Fisher Scientific) was added to each sample (Kuhnert et al., 2019). The DNA was eluted in 60 µl H₂O and stored at –20 °C until further processing.

2.3. PCR assays

Primers and probes used for the previously published PCR assays are summarized in Table S1. For detection of BDD associated *Treponema* spp. the nested PCR of Evans et al. (2009) was applied to biopsy samples, detecting the three phylogroups represented by the species *T. phagedenis*, *T. pedis* and *T. medium*. Amplicons were visualized on agarose gels. Corresponding positive controls were based on genomic DNA from the type strains *T. medium* ATCC 700293^T, *T. phagedenis* B43.1^T and *T. pedis* DSM 18691^T. Both strands of PCR amplicons were Sanger sequenced (Microsynth, Balgach, Switzerland) using corresponding PCR primers followed by Blast analysis (blast.ncbi.nlm.nih.gov) to confirm species.

For detection of virulent (*aprV2*-positive) and benign (*aprB2*-positive) *D. nodosus* the real-time PCR of (Stauble et al., 2014a) with adaptation by Kuhnert et al. (2019) was applied to swab as well as biopsy samples. Positive controls were based on genomic DNA of *aprV2*-positive *D. nodosus* ATCC 25549^T and *aprB2*-positive *D. nodosus* JF5922.

Detection of *F. necrophorum* was achieved by the real-time PCR of Jensen et al. (2007) and included genomic DNA of CCUG 9994^T and CCUG 42162^T as positive controls for *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme*, respectively. Specimens investigated for the presence of *F. necrophorum* were swabs and biopsies.

All negative PCR controls were based on H₂O as template.

To check for correct real-time PCR performance the Xeno LIZ Primer Probe Mix (Thermo Fisher Scientific) was added to the PCR reaction mixes to amplify the IPC DNA. All samples were analyzed in duplicate, and samples were defined positive if both duplicates showed a Ct-value <40 in the specific reaction.

2.4. Genetic analysis of Swiss zebu cattle and comparison to other breeds

To assess the genetic background of Swiss zebu cattle, blood samples of 15 animals from farm A (n=7) and farm B (n=8) were sequenced and compared to publicly available sequences from four zebu breeds from South China (n=7), six zebu breeds from India-Pakistan (n=9), and Holstein cattle (n=21) as a *Bos taurus* representative. Sample accession numbers are listed in Table S2. Whole-genome sequence data were generated and analyzed as described in Démoulin et al., (2024). Briefly, raw reads were trimmed and filtered with fastp (Chen et al., 2018) version 0.23.2 and mapped to the bovine reference genome (ARS-UCD1.3 including the Btau5.0.1 Y chromosome) with bwa-mem2 (Vasimuddin et al., 2019) version 2.2.1 using the mem algorithm. Picard tools (<https://broadinstitute.github.io/picard>) version 3.0.0 was used to coordinate-sort mapped reads and to mark duplicates. Genotypes were called with GATK (McKenna et al., 2010) version 4.4.0.0, applying the GVCF workflow after recalibration of base quality scores. For Holstein samples, recalibration was based on known *Bos taurus* variants obtained from Ensembl release 109. As such variants were not available for *Bos indicus*, variants of all zebu samples were first identified without recalibration, hard-filtered, and used together with known *Bos taurus* variants from Ensembl release 109 for recalibration before the final round of genotype calling. Biallelic SNPs were extracted and hard-filtered according to the GATK recommendations separately for Holstein and zebu data sets, and then combined using BCFtools (Danecek and McCarthy, 2017) version 1.18 by setting sites that were missing in one data set to the reference genotype. After retaining only autosomal SNPs with a minor allele frequency above 0.02 and no missing genotypes, SNPs were pruned for linkage equilibrium with PLINK2 (Chang et al., 2015) version 2.0.0a3.3. Principal component analysis (PCA) on the genetic relationship matrix obtained from the remaining 2'878'636 SNPs was performed with GCTA (Yang et al., 2010; Yang et al., 2011) version 1.94.1.

2.5. Statistical analysis

Statistical significance between prevalence of specific bacterial species of zebu and taurine cattle was calculated using Fisher's exact test with a value of significance $p < 0.05$.

3. Results

None of the zebu nor taurine cattle feet investigated in this study showed signs of BDD or any other lesions by visual inspection.

All biopsies obtained from the zebu (n=49) tested negative for *Treponema* spp. associated with BDD (Table 1). In the taurine cattle set 7 animals (35 %) could be confirmed by sequencing to harbor *T. phagedenis* while the other two, *T. pedis* and *T. medium*, were not observed (Table 1). Accordingly, 7 farms (41 %) were confirmed to harbor *T. phagedenis*.

All animals, zebu as well as taurine cattle, were negative for the virulent (*aprV2*-positive) *D. nodosus* (data not shown) in all specimens tested. The benign (*aprB2*-positive) strain of *D. nodosus* was detected in 14 of the 60 zebu (23 %) and in 18 of the 20 taurine cattle (90 %). On a

Table 1

Specimens from zebu (*Bos indicus*) and taurine cattle (*Bos taurus*) investigated in this study and results obtained from detection of ruminant hoof pathogens *Treponema* spp., *Dichelobacter nodosus* (*aprB2*), and *Fusobacterium necrophorum* subspecies by PCR in these specimens.

Farm	Animal	Date	swab		biopsy		
			<i>aprB2</i>	<i>F.nec.subsp.</i>	<i>aprB2</i>	<i>F.nec.subsp.</i>	<i>Treponema</i> spp.
A (BE)	f, cow	24.11.2021	na	na	-	-	-
	f, stirk	24.11.2021	na	na	-	-	-
	ox	24.11.2021	na	na	-	-	-
	f, cow	24.11.2021	na	na	-	-	-
	f, cow	24.11.2021	na	na	(+)	-	-
	f, cow	12.01.2022	-	-	na	na	na
	f, cow	12.01.2022	-	-	na	na	na
	f, cow	12.01.2022	-	-	na	na	na
	f, cow	12.01.2022	-	-	na	na	na
	f, cow	12.01.2022	-	-	na	na	na
	f, stirk	12.01.2022	-	-	na	na	na
	f, cow	26.10.2022	-	-	-	-	-
	m, stirk	26.10.2022	-	-	-	-	-
	m, stirk	26.10.2022	-	-	-	-	-
	m, stirk	26.10.2022	-	-	-	-	-
	f, cow	18.01.2023	-	-	-	-	-
	f, cow	18.01.2023	-	-	-	-	-
	m, stirk	08.02.2023	-	-	-	-	-
	m, stirk	08.02.2023	-	-	-	-	-
	m, stirk	08.02.2023	-	-	-	-	-
	f, cow	08.02.2023	-	-	-	-	-
	f, cow	08.02.2023	-	-	-	-	-
	f, cow	05.04.2023	-	-	-	-	-
	f, cow	05.04.2023	-	-	-	-	-
B (SO)	m, stirk	04.04.2022	+	-	+	-	-
	m, stirk	04.04.2022	(+)	-	-	-	-
	m, stirk	04.04.2022	-	-	+	-	-
	m, stirk	04.04.2022	(+)	-	(+)	-	-
	f, cow	06.04.2022	-	-	(+)	-	-
	f, stirk	06.04.2022	(+)	-	(+)	-	-
	f, cow	20.04.2022	-	-	na	na	na
	f, stirk	20.04.2022	(+)	-	na	na	na
	m, stirk	20.04.2022	(+)	-	na	na	na
	m, bull	20.04.2022	(+)	-	na	na	na
	f, cow	20.04.2022	-	-	na	na	na
	C (SO)	m, calf	24.02.2022	-	-	-	-
f, cow		24.02.2022	-	-	-	-	-
D (BE)	m, stirk	21.03.2022	+	-	+	-	-
	m, stirk	25.04.2022	+	-	+	-	-
E (BE)	f, stirk	25.05.2022	(+)	-	(+)	-	-
	ox	08.06.2022	(+)	-	+	-	-
	m, stirk	02.11.2022	-	-	-	-	-
F (OW)	f, stirk	02.11.2022	-	-	-	-	-
	m, bull	19.09.2022	-	-	-	-	-
	m, stirk	19.09.2022	-	-	-	-	-
	m, stirk	19.09.2022	-	-	-	-	-
	m, stirk	19.09.2022	-	-	-	-	-
	m, stirk	07.11.2022	-	-	-	-	-
G (AG)	stirk	21.09.2022	-	-	-	-	-
	stirk	21.09.2022	-	-	-	-	-
H (BE)	m, stirk	04.10.2022	-	-	-	-	-
	m, stirk	04.10.2022	-	-	-	-	-
	f, cow	11.10.2022	-	-	-	-	-
	m, stirk	11.10.2022	-	-	-	-	-
	f, cow	18.10.2022	-	-	-	-	-
	f, stirk	18.10.2022	-	-	-	-	-
	m, stirk	18.10.2022	-	-	-	-	-
	f, cow	25.10.2022	-	-	-	-	-
	f, cow	25.10.2022	-	-	-	-	-
	m, stirk	25.10.2022	-	-	-	-	-
Farm	Animal	Date	swab <i>aprB2</i>	<i>F.nec.subsp</i>	biopsy <i>aprB2</i>	<i>F.nec.subsp.</i>	<i>Treponema</i> spp.
1	f, cow	30.01.2023	-	-	-		-
	f, cow	30.01.2023	+	-	+		+ <i>T. phagedenis</i>
	m, stirk	06.02.2023	+	-	+		-
2	m, bull	13.02.2023	+	-	-		-
	f, stirk	13.02.2023	+	-	+		-
3	f, stirk	30.01.2023	-	-	-		-
4	m, stirk	30.01.2023	+	-	+		-
5	m, stirk	30.01.2023	+	-	+		+ <i>T. phagedenis</i>
6	m, stirk	30.01.2023	+	-	+		-
7	f, stirk	06.02.2023	+	-	+		-

Table 1 (continued)

Farm	Animal	Date	swab		biopsy		
			<i>aprB2</i>	<i>F.nec.subsp.</i>	<i>aprB2</i>	<i>F.nec.subsp.</i>	<i>Treponema spp.</i>
8	f, cow	06.02.2023	+	-	+		-
9	m, stirk	06.02.2023	+	(+) <i>fundiliforme</i>	+	+ <i>fundiliforme</i>	-
10	m, stirk	13.02.2023	+	-	+		(+) <i>T. phagedenis</i>
11	m, calf	13.02.2023	+	-	+		+ <i>T. phagedenis</i>
12	f, cow	15.02.2023	+	-	+		-
13	f, cow	15.02.2023	+	-	+		-
14	f, cow	15.02.2023	+	+ <i>necrophorum</i>	+	(+) <i>necrophorum</i>	+ <i>T. phagedenis</i>
15	f, cow	15.02.2023	+	+ <i>fundiliforme</i>	+	(+) <i>fundiliforme</i>	+ <i>T. phagedenis</i>
16	m, stirk	15.02.2023	+	-	+		+ <i>T. phagedenis</i>
17	m, bull	15.02.2023	+	-	+		-

na: not available; +: both feet PCR positive; (+): only one of the two feet PCR positive; -: PCR negative.
f: female; m: male; AG: Aargau; BE: Bern; SO: Solothurn; OW: Obwalden
f: female; m: male

farm level 50 % of zebu farms (including the crossbreed farm) had positive animals, while 94 % of the taurine cattle farms were positive for benign *D. nodosus*. However, in farm A only a single foot out of 24 animals was positive. In general, with zebu in most cases only a single foot

revealed positive for *D. nodosus* while in taurine cattle always both feet were clearly positive (Table 1). In agreement to that, mean Ct-values observed with purebred zebu were much higher (34.5) than those seen with taurine cattle (25.9) or the crossbred animals (24.9) indicating

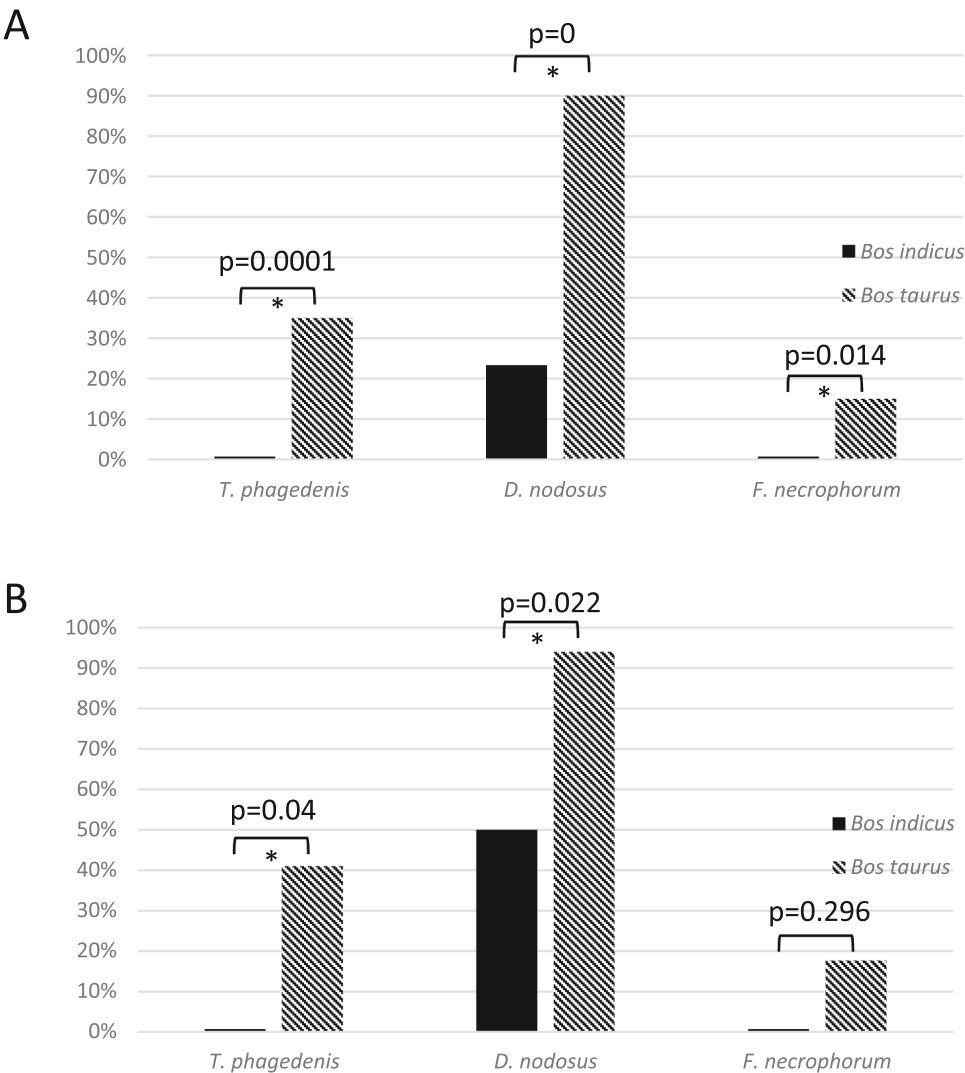


Fig. 1. Prevalence of ruminant hoof pathogens *Treponema phagedenis*, *Dichelobacter nodosus* and *Fusobacterium necrophorum* determined by PCR. The prevalence on the y-axis is given in percentage while the x-axis indicates the corresponding pathogens. Panel A: Prevalence of the three ruminant foot pathogen in zebu (*Bos indicus*) and taurine cattle (*Bos taurus*) at single animal level. Panel B: Prevalence of the three ruminant foot pathogen in zebu (*Bos indicus*) and taurine cattle (*Bos taurus*) at farm level. The asterisks indicate significant differences based on calculated p-values.

a roughly 500-fold higher bacterial load in the latter two.

The difference in prevalence of *Treponema* spp. and *D. nodosus* between cattle and zebu was significant, overall as well as at the farm level (Fig. 1). The third hoof pathogen tested for, *F. necrophorum*, was detected in three taurine cattle (15 % at animal and 18 % at farm level) with two harboring the subsp. *fundiliforme* and the third the subsp. *necrophorum* (Table 1). The difference in prevalence of this pathogen between zebu and taurine cattle was significant at the animal level.

By investigating the genetic relationship between *Bos taurus* and *Bos indicus* by PCA, a clear separation of Swiss zebu cattle from *Bos taurus* was observed, together with clustering close to *Bos indicus* breeds from India-Pakistan (Fig. 2). Moreover, Swiss zebu showed a relatively high genetic diversity within and between farms.

4. Discussion

Here we report to the best of our knowledge the first time resistance of zebu towards colonization by major ruminant hoof pathogens. The primary focus was on *Treponema* spp. associated with BDD, a disease that emerged in Switzerland over the past 20 years with prevalence at the cow level increasing between 2002 and 2021 from 5 % to 21 % (Fürmann et al., 2024). None of the *Treponema* spp. was detected in zebu while 35 % of taurine cattle and 41 % of taurine cattle farms were positive for *T. phagedenis* (Kuhnert et al., 2020), a highly significant difference between the two. It must be mentioned that the cattle feet were randomly collected without any of the feet showing typical BDD lesions. Moreover, most of the animals were also from suckler cow husbandry making this sample set comparable to the one of zebu. Like in previous studies, there was a high prevalence of benign *D. nodosus* in taurine cattle with 90 % of animals and even 94 % of farms being positive. Again, we observed a significant difference to zebu in that respect. This difference was more pronounced at the animal level than at the farm level (Fig. 1). However, there are some confounding factors to be considered here. From farm A a single animal out of 24 tested weakly positive on only one of the two feet. The animals from this farm were slaughtered together with taurine cattle, and contamination could occur

occasionally in the waiting area, where other cattle were kept before slaughter. In addition, this animal was kept in a mixed herd one month before joining the zebu herd. Given the one weakly positive animal from this herd with the highest number of tested animals the farm could be grouped as negative as well. With farm B a special situation led to the numerous positive animals. The zebras were kept in a free stall together with goats and to leave and enter the stable they had to pass the part of the stable where the goats were kept. The goats had enormous problems with footrot and they tested strongly positive for benign *D. nodosus*. Again, even so, zebras were only weakly positive with a mean Ct-value > 36. Therefore, the positivity of the zebu feet resulted most likely from a contamination of feet by the massively *D. nodosus*-shedding goats. The two animals of farm D were crossbreeds of zebu and Limousin cattle. Both animals were strongly positive in the real-time PCR with Ct-values in the same range as those of taurine cattle, indicating colonization rather than contamination. This could be an indication, that certain crossbreed loose resistance towards colonization by specific pathogens, in particular *D. nodosus* in this case. A special situation was also observed with farm E. This farm had purebred zebu which were held during spring/summer on the meadow of farm D. Two animals slaughtered during that time were weakly positive for benign *D. nodosus*, most probably because of feet contamination resulting from sharing the meadow with the strongly positive crossbred animals. Interestingly, the two animals slaughtered in November, when the animals were already back on the purebred zebu farm E for two months, tested negative. Despite this bias (3 *D. nodosus* negative farms defined as positive due to cross-contamination, one crossbreed farm included) the difference in prevalence of the two pathogens between zebu and taurine cattle was highly significant.

Finally, the results from *F. necrophorum* indicate a lower prevalence of this bacterium in Swiss cattle and lower as e.g. the 32 % of healthy cattle being positive for *F. necrophorum* in a UK study (Sullivan et al., 2015). However, no data was available up to now for Switzerland and the difference to zebu, which were all negative is significant at the animal level.

Analysis of genomic data showed clear separation of Swiss zebu from taurine cattle and clustering with other zebu breeds, indicating no or at most small amounts of genomic admixture with *Bos taurus*. The observed clustering of Swiss zebu close to *Bos indicus* breeds from India-Pakistan is consistent with findings for German zebu based on mitochondrial DNA (Pramod et al., 2019). Furthermore, the relatively high genetic diversity of Swiss zebu, particularly when compared to Holstein, suggests that inbreeding remains low despite their small population size in Switzerland.

Concluding, zebu is more resistant to colonization by bacterial hoof pathogens than taurine cattle. *F. necrophorum* was not found in zebu and showed the lowest prevalence of the three in taurine cattle. *D. nodosus* seems to be unable to colonizing zebu in contrast to taurine cattle including crossbreeds. The fact, that no virulent *D. nodosus* was detected in zebu nor in taurine cattle confirms earlier findings that cattle do not pose a risk of reservoir possibly hampering the planned Swiss ovine footrot control program. The most intriguing result is the absence of *T. phagedenis* in zebu known to be involved in BDD, which corroborates the experience that the disease has so far not been encountered in Swiss zebu. The fact that such *T. phagedenis* were also absent in the specific crossbreed animals could be a promising indication, that such breeds would be resistant towards BDD as well. However, more studies including various crossbreeds are needed to confirm this observation.

CRedit authorship contribution statement

Dorothea Lindtke: Writing – review & editing, Validation, Methodology, Formal analysis. **Joerg Jores:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization. **Nadia Loosli:** Writing – review & editing, Methodology, Conceptualization. **Isabelle Brodard:** Writing – review & editing,

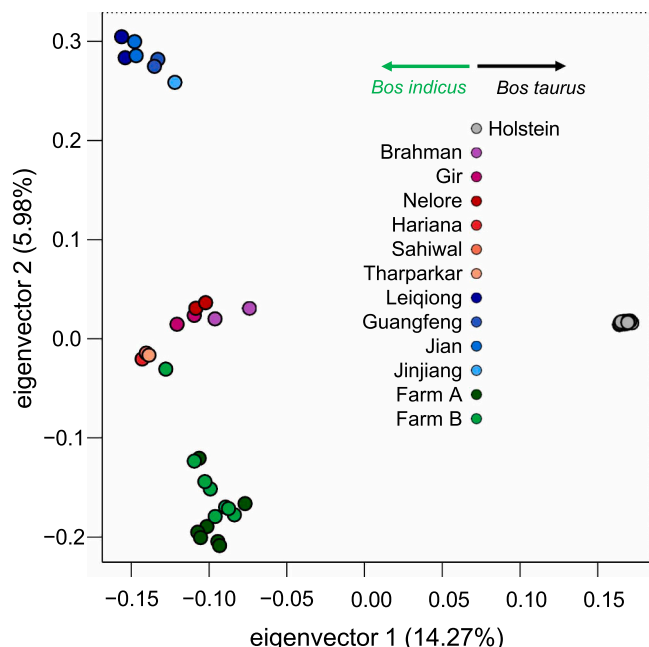


Fig. 2. First and second principal components (eigenvector 1 and 2) estimated from whole-genome sequencing data by principal component analysis (PCA). *Bos indicus* breeds from South China are shown in blue, from India-Pakistan in orange, red and purple, and from Switzerland in green. Holstein was used as a *Bos taurus* representative and is shown in gray.

Methodology. **Peter Kuhnert:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of Competing Interest

None

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.vetmic.2024.110184](https://doi.org/10.1016/j.vetmic.2024.110184).

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