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Biodiversity and microbial safety of artisanal Malian sour milk *fènè* and development of adapted starter cultures for controlled production

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Stephan Markus Wullschleger

Dipl. Lm-Ing., ETH Zurich

Born February 4, 1980

Citizen of

Aarburg (AG)

Accepted on the recommendation of

Prof. Dr. Christophe Lacroix, examiner

Prof. Dr. Leo Meile, co-examiner

Prof. Dr. Bassirou Bonfoh, co-examiner

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Preface

Ancient and extensively practiced agricultural traditions in Mali and concomittant high demand on milk and sour milk for the daily diet are the main forces behind sustainable development of the local dairy market and related research projects. The chance of success of applied research projects in developing countries is greater if such projects are executed in collaboration with local organizations and leading research partners combining basic research and local application. Local organizations should propose research questions where they have the detailed knowledge of local needs and conditions indicating local limits for state of the art basic research and application of achieved results in the field. Their research partner(s) may then contribute with state of the art knowledge and infrastructure to address the research question and finally achieve high impact publications and local application of the results leading to globally recognized research projects. Exchange programs of committed researchers are the key to guaranteeing long term application, knowledge transfer and trust to collaborating organizations favoring follow-up or new research projects.

I am very happy that we got the full support of all collaborating organizations behind this research project, the serendipitous exchange program between the Laboratoire Central Vétérinaire and ETH Zurich and final application experiments at small-scale production. This project is a good example of international collaboration combining basic research and application on the field of achieved results leading to new research projects - current under evaluation and local business opportunities ready to apply new knowledge.

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Abbreviations

ABR	antibiotic resistant bacteria
AD	anno Domini
AR	antibiotic resistance
ARDRA	amplified ribosomal DNA restriction analysis
ATCC	the American Type Culture Collection
BAL	bactéries acido lactiques
BC	before Christ
BFM	basic *fènè* microflora
Bp	base pairs
BP	Baird Parker
CCM	Czech collection of microorganisms
CCUG	culture collection, university of Göteborg, Sweden
cfu	colony forming units
CLSI	clinical and laboratory standards institute, Wayne, USA
CMI	concentration minimal d'inhibitation
CSBSE	complexe *St. bovis* / *St. equinus*
D2S	defined two-species starter culture
DMS	difined multi-species starter culture
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate (dATP, dGTP, dCTP and dTTP)
DSMZ	German collection of microorganisms and cell cultures, Braunschweig, Germany
E.	*Enterococcus* or *Escherichia*
e.g.	exempli gratia
ECOFF	epidemiological cut off value
EDTA	ethylenediaminetetraactic acid
EFSA	European food safety agency
EPS	exopolysaccharide
ETH Zurich	Eidgenössische Technische Hochschule (Swiss Federal Institute of Technology) Zurich, Switerland
EUCAST	European committee on antimicrobial susceptibility testing
FAO	food and agricultur organization of the United Nations, Rome, Italy
FBC	collection of the laboratory of food biotechnology, ETH Zurich, Switzerland
FDA	food and drug administration of the United States of America
FEEDAP	European food safety authority panael on additives and products or substances used in animal feed
FME	ferment défini multi-espèces
GDP	gross domestic product
GRAS	generally recognized as safe
i.e.	id est
INSAH	institut de Sahel, Bamako, Mali
kb	kilo base pairs
KBE	keimbildende Einheit
KF	azide maltose
KFPE	commission for research partnerships with developing countries, Bern, Switzerland
L.	*Listeria*
LAB	lactic acid bacteria

Lb.	*Lactobacillus*
Lc.	*Lactococcus*
LCV	laboratoire central vétérinaire, Bamako, Mali
LMG	laboratory for microbiology Gent
LSM	LAB susceptibility test medium
MHK	minimale Hemmstoffkonzentration
MIC	minimal inhibition concentration
MRS	Man Rogosa and Sharpe
MRSL	MRS lactose
MSB	Milchsäurebakterien
MSK	Multispeziesstarterkultur
NCCR	Swiss national centre of competence in research, Bern, Switzerland
nov.	novum
OD	optical density
P.	*Pediococcus*
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PSM-A	pediocicci suelective medium with ampicillin
QPS	qualified presumtion of safety
rep-PCR	repetitive extragenic palindromic PCR
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
S.	*Staphylococcus*
sBFM	suplemented BFM
SBSEC	*St. bovis* / *St. equinus* complex
SBSEK	*St. bovis* / *St. equinus* Komplex
SGLH	Schweizerische Gesellschaft für Lebensmittelhygiene, Zürich, Schweiz
SM	*Streptococcus macedonicus* medium
SM_{tet}	SM with tetracycline
sp.	species
SSM	*Streptococcus thermophilus* susceptibility test medium
St.	*Streptococcus*
subsp.	subspecies
^{T}	type strain
TCs	transconjugants
ufc	unités formamt des colonies
V.	*Vagococcus*
VRBD	violet red bile dextrose
W.	*Weissella*

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Summary

Background:

Ancient Malian pastoral practices are evolving; traditional production of *fènè*, a spontaneously fermented sour milk, is moving toward commercial manufacture and already provides a substantial foundation for developing local economies. This economic boon through commercialization of traditional raw milk and dairy products, a major source of proteins in Malian diets, is accompanied unfortunately with a commensurate risk to human health due to the presence of putative human pathogens and the lack of standardized hygienic processes in domestic and commercial *fènè* production. This is further complicated by the absence of sufficient commercial logistics; controled transport and storage of milk and perishable foodstuffs. Farmers cannot sell their milk in a timely manner locally and the commercial local dairy industry still in its infancy is unable to process the milk safely. Sustainable production of safe dairy products, with regard to the spontaneous *fènè* fermentation process, which has not been investigated until now, will bring healthier food to market and regular income to farmers.

Objectives:

Elucidation of seasonal and process-driven variation of bacterial strain diversity of *fènè* microbiota during spontaneous fermentation was the goal of the first part of this thesis work. The practical application of this data through development of an adapted starter culture improving reproducible quality, establishing hygienic production standards eliminating opportunistic pathogens, decreasing antibiotic resistant bacteria and increasing food safety were the goals set in the latter half of this thesis.

Results:

A simple and powerful strategy for rapid, unequivocal identification/quantification of individual bacterial strains has proven itself invaluable in our primary objective of determining the complex commensal *fènè* flora composition as well as elevated levels of putative human pathogens within complex food matrices later on. The diversity of native *fènè* microbiota was assayed during three seasons (hot, cold and rainy) at one domestic production site and one small-scale dairy. A total of 1583 catalase-negative, Gram-positive bacteria were isolated from 98 samples. Enterococci and streptococci dominated the increased biodiversity range of lactic acid bacteria (LAB) evidenced by

[isolates/strain numbers]: *Lactobacillus fermentum* (77/18), *Weissella confusa* (75/21), *Lactococcus lactis* subsp. *lactis* (62/21), *Pediococcus pentosaceus* (36/30), *Lactobacillus plantarum* (24/9), *Weissella paramesenteroides* (11/3), *Lactococcus garvieae* (11), *Lactobacillus* sp. (10) and *Pediococcus acidilactici* (8/4).

Production-type and seasonal influences on strain diversity and bacterial populations were observed and recorded. Of the total 126 strains; only four were isolated repeatedly in two out of three seasons and a maximum of seven strains were found consistently in any single season at domestic and small-scale production. Highest bacterial counts for presumptive lactobacilli, lactococci and enterococci were found with 9.2±0.5, 9.2±0.3 and 8.8±0.3 Log_{10} colony forming unit (cfu)/ml during hot and lowest with 7.9±0.5, 8.2±0.8 and 7.8±1.0 Log_{10}(cfu/ml) during rainy season, respectively. No impact of the type of production was found on bacterial populations except for presumptive *Enterobacteriacea*-counts, with lowest counts of 7.1±0.4 Log_{10}(cfu/ml) at small-scale production.

Presumptive *Enterobacteriaceae* (6.5 - 9.0 Log_{10}(cfu/ml)) and *Staphylococcus aureus* (5.2±0.9 Log_{10}(cfu/ml)), pose a significant human health hazard. This treat was further corroborated by the identification of 596 enterococci and 454 streptococci from 1583 isolates. The identification of *Streptococcus infantarius* subsp*. infantarius* as putative dominante streptococci species with the conspicuous absence of *St. thermophilus* – usually occupying a central fermentative role – pointed towards spontaneously fermented *fènè* as a likely source for human infection and transmission.

Bacteria of the *St. bovis* / *St. equinus* complex (SBSEC) were the dominant species identified and main acidifier in spontaneous *fènè* fermentation. 29 traditional sour milks collected in the circle of Bamako (Mali), Kenya and Somalia were investigated on the presence of SBSEC bacteria and on their acquired antibiotic resistances. 95 *St. infantarius* subsp. *infantarius* and 26 St*. gallolyticus* subsp. *macedonicus* were isolated from 29 samples and identified by PCR, RFLP and rep-PCR to strain level. 13 of 20 selected SBSEC bacteria isolated from different products displayed resistance to tetracycline and harbored either the *tet*(M), *tet*(L) or *tet*(S) gene. Five of these strains displayed multidrug resistance against ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, neomycin, penicillin, streptomycin and tetraycline and only five

were susceptible to all 11 tested antibiotics. Combined erythromycin and tetracycline resistance of two *St. gallolyticus* subsp. *macedonicus* strains was related to the *erm*(B) and the *tet*(M) genes.

118 enterococci and 84 non-*Enterococcus* lactic acid bacteria (LAB) were tested on antibiotic susceptibility. The antibiotic resistance prevalence against tetracycline and oxytetracycline was directly linked to the amount of oxytetracycline purchased by farmers during the preceding season, with 40%, 26% and 17% tetracycline resistant enterococci found during cold, hot and rainy seasons, respectively. Tetracycline resistant enterococci harbored the *tet*(L), *tet*(M) and *tet*(S) resistance genes. One enterococci strain transferred its *tet*(L) and *tet*(M) genes to the donor strain *E. faecalis* JH-2-2 with a transfer rate of $1.9*10^{-6}$ transconjugants per donor. Tetracycline resistance was also predominant with 17% among non-*Enterococcus* LAB and was induced by the *tet*(M) gene in one *Lb. plantarum* and by the *tet*(S) gene in four *Lc. lactis* subsp. *lactis* and seven *St. infantarius* subsp. *infantarius*.

Ten isolates among the 1583 isolates of *fènè* microbiota belong to four strain clusters of the novel species *Vagococcus teuberi* sp. nov. *V. teuberi* was phenotypic resistant to tetracycline (minimal inhibition concentration (MIC) 64 µg/ml) and trimethoprim (MIC 256 µg/ml), but harbored only the trimethoprim resistance gene *dfr(G)*.

The screening of 51 antibiotic susceptible LAB strains on acidification properties, antimicrobial activity and exopolysaccharide production led to the development of a defined two-species starter culture consisting of *St. thermophilus* (industrial culture to replace *St. infantarius* subsp. *infantarius*) *and Lb. plantarum* and a defined multi-species starter culture (DMS) consisting of *St. thermophilus*, *Lb. fermentum*, *Lb. plantarum*, *Lc. lactis* subsp. *lactis* and *W. paramesenteroides*. Both cultures showed high antimicrobial activity against *Listeria ivannovii* HPB28 and *Enterococcus faecalis* DSM 2048^T at 30℃ and 35℃ as well as high acidification and stability during five back-slopping cultures at 35℃ and 40℃. The DMS and an industrial *St. thermophilus* starter culture were applied at ambient temperature (27-36℃) in small-scale fermentations in Mali. Fastest acidification was found for the DMS, reaching pH 4.8±0.2 within 9 hours.

Conclusion:

The microbial composition of spontaneous *fènè* fermentation processes was dependent on season, environment of production-type and showed elevated biodiversity of lactic acid bacteria. Opportunistic pathogens are predominant in *fènè* microbiota beside lactobacilli, lactococci, pediococci and *Weissella* and the key microorganism for spontaneous *fènè* fermentation is the animal and human pathogen *St. infantarius* subsp. *infantarius*. The *fènè* microbiota composes a gene pool of transferable antibiotic resistance genes, of which the same tetracycline resistance genes are found among different genera. Recontamination of heat-treated milk during fermentation is reduced but not eliminated with the application of starter cultures. Food safety and quality of *fènè* can be improved through standardized and temperature-controlled fermentation processes, rigorous hygienic control and the ban of uncontrolled antibiotic use in animal husbandry.

Summerized, we analyzed the first time growth dynamics and strain fulctuations of spontaneous *fènè* fermentation processes, descripted the novel species *V. teuberi* sp. nov., determined a remarkably high tetracycline gene pool, and finally developed a safe and adapted starter culture, which’s application has been proven at small-scale production in rural Mali.

Zusammenfassung

Hintergrund:

Mali hat eine ausgeprägte landwirtschaftliche Tradition, die immer noch auf extensiven Produktionssystemen basiert obwohl Milch ein wichtiger Eiweisslieferant in der lokalen Ernährung darstellt und für die nationale Wirtschaft von grosser Bedeutung ist. Die traditionelle Herstellung von *fènè* – eine spontan fermentierte Sauermilch – entwickelt sich in Richtung kommerzielle Produktion und bildet eine wesentliche Basis für die wirtschaftliche Entwicklung. Leider weist einheimische Milch, die auf dem lokalen Markt informell verkauft wird, eine ungenügende hygienische Qualität auf. Dies weil die Bauern ihre Milch nicht täglich verkaufen können, und keine national organisierte Milchindustrie die Milch verarbeitet. Eine wirtschaftlich nachhaltige Produktion von mikrobiologisch einwandfreien Milchprodukten, mit speziellem Bezug auf den spontanen *fènè* Fermentierungsprozess, welcher bis jetzt noch nicht untersucht wurde, würde die Bevölkerung mit sicheren und somit gesünderen lokalen Nahrungsmitteln versorgen und den Bauern ein regelmässiges Einkommen ermöglichen.

Ziele:

Als erstes Ziel wurde die Mikroflora von Sauermilchfermentationsprozessen analysiert und untersucht, ob die Produktionsart oder die Jahreszeit einen Einfluss auf die bakterielle Zusammensetzung der Fènèmikroflora hat. Das Hauptziel war anschliesslich das Entwickeln einer sicheren und adaptierten Starterkultur, mit welcher qualitativ ausgezeichneter und mikorbiell sicherer *Fènè* hergestellt werden kann. Dafür wurden Sicherheitsaspekte, wie Antibiotikaresistenz in Bakterien oder opportunistisch pathogene Bakterien genau untersucht.

Resultate:

Die komplexe symbiotische Fènèmikroflora, potentielle humanpathogenen Bakterien und eine neue Bakterienspezies konnten mit einer einfachen aber wirkungsvollen Typisierungsstrategie auf Stammesniveau bestimmt werden. Die Diversität der bakteriellen Fènèflora wurde während den drei Jahreszeiten, d.h. der heissen Jahreszeit, der kalten Jahreszeit und der Regenzeit in Mali untersucht. Die dafür benötigten Proben wurden auf einem Familienhof und in einer Kleinmolkerei gesammelt. Total wurden 1583 Gram-positive und katalase-negative Bakterien von 98 Proben isoliert. Die Genera Enterokokkus und Streptokokkus dominierten über eine Mikroflora

die eine hohe Biodiversität an folgenden Milchsäurebakterien (MSB) aufwies [Anzahl Isolate / Anzahl Stämme]: *Lactobacillus fermentum* (77/18), *Weissella confusa* (75/21), *Lactococcus lactis* subsp. *lactis* (62/21), *Pediococcus pentosaceus* (36/30), *Lactobacillus plantarum* (24/9), *Weissella paramesenteroides* (11/3), *Lactococcus garvieae* (11), *Lactobacillus* sp. (10) and *Pediococcus acidilactici* (8/4). Dabei konnte gezeigt werden, dass bakterielle Verunreinigungen von der Umgebung der Kleinmolkerei in die erhitzte Milch gelangen und somit die spontane Fermentation der Milch zu *Fènè* verursachen.

Die Bakterienpopulation sowie die Biodiversität der Bakterien auf Stammesniveau wurden durch die Jahreszeiten und die unterschiedlichen Produktionsverfahren beeinflusst. Von total 126 bestimmten Stämmen wurden nur vier Stämme in mindestens zwei Jahreszeiten und sieben Stämme in der Fènèproduktion auf dem Familienhof und der Kleinmolkerei gefunden.

Die höchsten Bakterienzahlen wurden für vermutliche Laktobazillen, Laktokokken und Enterokokken mit jeweils 9.2±0.5, 9.2±0.3 and 8.8±0.3 Log_{10} keimbildender Einheiten (KBE)/ml in der heissen Jahreszeit und die tiefsten mit jeweils 7.9±0.5, 8.2±0.8 and 7.8±1.0 Log_{10}(KBE/ml) in der Regenzeit gefunden. Die Bakterienzahlen wurden nicht von der Produktionsart beeinflusst, ausser, dass die Zahlen von vermutlichen *Enterobacteriacea* mit 7.1±0.4 Log_{10}(KBE/ml) in *Fènè,* welcher in der Kleinmolkerei produziert wurde, am tiefsten waren.

Vermutliche *Enterobacteriaceae* (6.5 - 9.0 Log_{10}(KBE/ml)) und *Staphylococcus aureus* (5.2±0.9 Log_{10}(KBE/ml)) deuten auf ein hohes Gesundheitsrisiko für die Bevölkerung hin. Diese Gefahr wurde erhärtet, nachdem von den 1583 Isolaten aus der Fènèmikroflora 596 als Enterokokken und 454 als Streptokokken identifiziert wurden. Die Identifizierung von *Streptococcus infantarius* subsp. *infantarius* als mögliche dominierende Streptokokken Spezies und die überraschende Absenz von *St. thermophilus,* welcher gewöhnlich eine zentrale Rolle in der Sauermilchproduktion einnimmt, legen nahe, dass spontan fermentierter *Fènè* eine mögliche Übertragsungsquelle von menschlichen Infektionserkrankungen darstellen kann.

29 Proben von traditionell hergestellter Sauermilch wurden in verschiedenen Dörfern in Mali, Kenia und Somalia gesammelt und auf das Vorkommen von Bakterien die zum *St.*

bovis / *St. equinus* Komplex (SBSEK) gehören und desseren aufgenommene Antibiotikaresistenzen untersucht. Es wurden 95 *St. infantarius* subsp. *infantarius* und 26 *St. gallolyticus* subsp. *macedonicus* Isolate mittels PCR, RFLP und rep-PCR bestimmt. 13 von 20 SBSEK Bakterien, welche von unterschiedlichen Proben isoliert wurden, waren tetracyclinresistenz und trugen die entsprechenden Resistenzgenen *tet*(L), *tet*(M) und *tet*(S). Fünf dieser Bakterien zeigten Multiresistenzen gegenüber Ampicillin, Chloramphenicol, Clindamycin, Erythromycin, Gentamicin, Neomycin, Penicillin, Streptomycin und Tetracycline und nur fünf dieser Bakterien waren empfindlich gegenüber alle 11 getesteten Antibiotika. Kombinierte Erythromycin und Tetracycline Resistenz von zwei *St. gallolyticus* subsp. *macedonicus* Stämmen basierte auf den Resistenzgenen *erm*(B) und *tet*(M).

118 Enterokokken und 84 nicht-Enterokokken MSB wurden auf ihre Antibiotikaempfindlichkeit getestet. Es konnte gezeigt werden, dass die Anzahl tetracyclineresistenter Bakterien in direktem Zusammenhang zu der Menge verkauftem Oxytetracycline in der vorangegangenen Jahreszeit stand. Es wurden jeweils 40%, 26% und 17% tetracycline resistente Enterokokken in der kalten und heissen Jahreszeit sowie der Regenzeit gefunden. Tetracyclineresistente Enterokokken besassen folgende Resistenzgene: *tet*(L), *tet*(M) und *tet*(S). Die Tetracyclineresistenz überwiegte mit 17% auch bei nicht-Enterokokken MSB. Dabei wurde die Tetracyclinresistenz bei einem *Lb. plantarum* durch das *tet*(M) Gen und bei vier *Lc. lactis* subsp. *lactis* und sieben *St. infantarius* subsp. *infantarius* durch das *tet*(S) Gen induziert.

Zehn von 1583 Isolaten der Fènèmikroflora gehörten zur neuen Spezies *Vagococcus teuberi* sp. nov. und konnten in vier verschiedene Stammgruppen eingeteilt werden. *V. teuberi* DSM 21459^{T} zeigte phenotypische Tetracycline- (minimale Hemmstoffkonzentration (MHK) 64 µg/ml) und Trimethoprimresistenzen (MHK 256 µg/ml), trug aber nur das Trimethoprimresistenzgen *dfr*(G).

51 antibiotikaempfindliche MSB-Stämme wurden auf ihre Ansäuerungseigenschaften, antimikrobielle Aktivität und Exopolysaccharidproduktion getestet. Schliesslich konnte eine Zweispeziesstarterkultur, bestehend aus *St. thermophilus* (Industriekultur um *St. infantarius* subsp. *infantarius* zu ersetzen) und *Lb. plantarum* und eine Multispeziesstarterkultur (MSK), bestehend aus *St. thermophilus*, *Lb. fermentum*, *Lb.*

plantarum, *Lc. lactis* subsp. *lactis* und *W. paramesenteroides,* entwickelt werden. Beide Kulturen zeigten hohe antimikrobielle Aktivität gegen *Listeria ivannovii* HPB28 und *Enterococcus faecalis* DSM 2048^T bei 30℃ und 35℃ sowie gute Ansäuerungseigenschaften und Stabilität während fünf „back-slopping" Kulturen bei 35℃ und 40℃. Die Anwendung der MSK sowie einer in dustriellen *St. thermophilus* Starterkultur wurde bei Umgebungstemperatur (27℃ – 36℃) in der Kleinmolkerei in Mali getestet. Die beste Ansäuerung zeigte die MSK und erreichte ein pH von 4.8±0.2 nach 9 h. Erneute bakterielle Verunreinigungen erhitzter und beimpfter Milch konnten durch den Einsatz von Starterkulturen nicht eliminiert aber gegenüber der traditionellen Fènèherstellung reduziert werden.

Schlussfolgerungen:

Die Zusammensetzung der Mikroflora von spontanen Fènèfermentierungsprozessen wurde durch die Jahreszeiten und die Produktionsarten beeinflusst und zeigte eine hohe Biodiversität. Die Fènèmikorflora wurde von mutmasslich pathogenen Bakterien dominiert und der tier- und humanpathogene *St. infantarius* subsp. *infantarius* stellte sich als Schlüsselorganismus in der Milchfermentation zu *Fènè* heraus. Im Weiteren stellt die Fènèmikroflora einen Genpool von transferierbaren Antibiotikaresistenzgenen dar, wovon dieselben Tetracyclineresistenzgene in verschiedenen Genera der Fènèmikroflora gefunden wurden. Erneute Verunreinigungen erhitzter Milch mit vermeintlich pathogenen Bakterien konnte mit dem Einsatz von Starterkulturen reduziert aber nicht verhindert werden. Das beste Ansäuerungsverhalten zeigte die neu entwickelte Multispeziesstarterkultur in Laborexperimenten und der Fènèproduktion in der Kleinmolkerei in Mali. Die Lebensmittelsicherheit und Produktequalität von *Fènè* könnte stark erhöht werden, indem standardisierte und temperaturkontrollierte Fermentationsprozesse in Kleinmolkereien implementiert würden, rigorose Hygienemassnahmen eingeführt würden und der unkontrollierte Einsatz von Antibiotika in der Viehwirtschaft verhindert würde.

Wir analysierten das erste Mali Wachstumsdynamik und Stammfluktuationen im spontanenen *fènè* Fermentationsprozess, beschrieben die neue Spezies *V. teuberi* sp. nov., bestimmten einen sehr hohen tetracyclineresistanz Genpool und schliesslich entwickelten eine sichere und angepasste Starterkultur, deren Anwendung für die Kleinproduktion im ländlichen Mali gezeigt werden konnte.

Résumé

Fond:

L'agriculture malienne est encore aujourd'hui basée sur un système de production extensive et est d'une grande importance pour l'économie du pays. Le lait est généralement consommé sous forme de lait fermenté spontanément (*fènè*), et jour un rôle primordial dans la vie sociale et économique. Le lait est la principale source de protéines dans l'alimentation quotidienne des Maliens. Les laits produits localement et vendus dans les marchés locaux ont malheureusement une qualité hygiénique médiocre. En effet les producteurs ou bergers ne peuvent pas vendre quotidiennement leur lait car il n'existe pas d'industrie laitière qui transforme le lait local au Mali. Une production durable des produits laitiers sains en termes de la qualité microbienne, surtout dans le procès de la fermentation spontanément du *fènè,* qui n'est pas du tout étudier, conféra à une alimentation saine pour les consommateurs et des revenus réguliers aux producteurs/bergers.

Objectifs:

Le premier l'objectif de cette étude était d'analyser l'effet des saisons et des processus de la fabrication du *fènè* sur la diversité des souches dans la microflore du *fènè* pendant la fermentation spontanée. Le second objectif était orienté sur le développement d'un ferment adapté pour la production de *fènè* de qualité sûre*.* Les aspects de la sécurité liés à la résistance des bactéries aux antibiotiques et aux bactéries opportunistes pathogènes sont analysés en détail.

Résultats:

Une stratégie simple et efficace pour la quantification et l'identification des souches bactériennes individuelles a été testée pour déterminer la composition complexe de la microflore symbiotique du *fènè,* et démontrer des niveaux élevés des bactéries poténtiellement pathogènes pour l'homme. Cela a permis la découverte d'espèces bactériennes nouvelles dans les matrices complexes d'aliment. La diversité de la microflore naturelle du *fènè* a été analysée pendant trois saisons (chaude, fraîche et hivernale) dans une production de taille familliale et dans une mini-laiterie. Un total de 1583 bactéries catalase-négative à Gram positife a été isolé de 98 échantillons de *fènè*. Les entérocoques et les streptocoques dominaient avec une biodiversité élevée des bactéries acido lactiques (BAL) identifiées avec [isolats/souches]: *Lactobacillus*

fermentum (77/18), *Weissella confusa* (75/21), *Lactococcus lactis* subsp. *lactis* (62/21), *Pediococcus pentosaceus* (36/30), *Lactobacillus plantarum* (24/9), *Weissella paramesenteroides* (11/3), *Lactococcus garvieae* (11), *Lactobacillus* sp. (10) et *Pediococcus acidilactici* (8/4).

L'équipement et l'environnement de la mini-laiterie sont les points d'entrée des contaminations bactériennes dans le lait préalablement chauffé utilisé pour la production du *fènè* dans la mini-laiterie. La saison et le type de production ont un effet significatif ($p<0.05$) sur la diversité des souches et les populations bactériennes. Seulement quatre souches a été isolées plusieurs fois pendant deux saisons et seulement sept souches ont été isolées de la production familale et de la production dans la mini-laiterie pendant la même saison sur un total de 126 souches. Le nombre de bactéries était plus élevé pour les lactobacilles, lactocoques et les entérocoques présumées étaient déterminés avec 9,2±0,5, 9.2±0,3 and 8,8±0,3 Log_{10} unités formant des colonies (ufc)/ml pendant la saison chaude et les nombres plus bas avec 7,9±0,5, 8,2±0,8 and 7,8±1,0 Log_{10}(ufc/ml) pendant la saison hivernale, respectivement. Le procédé de la fabrication du *fènè* n'a pas eu d'effet sur les populations microbiennes, sauf pour le nombre d'*Enterobacteriaceae* présumées avec moins de 7,1±0,4 Log_{10}(ufc/ml) dans la production de la mini-laiterie.

Le nombre d'*Enterobacteriaceae* présumées (6,5 - 9,0 Log_{10}(ufc/ml)) et de *Staphylococcus aureus* (5,2±0,9 Log_{10}(ufc/ml)), pendant la saison chaude indique un danger significatif pour la santé des consommateurs. Ceci est corroboré par l'identification de 596 entérocoques et 454 streptocoques comme populations dominantes. L'identification du *St. infantarius* subsp. *infantarius* comme espèce dominante présumée des streptocoques et l'absence frappante de *St. thermophilus* – normalement occupant un rôle central dans la fermentation des laits acidifiés – montre que le *fènè* fermenté spontanément peut être une source de transmission des infections pathogènes pour l'homme.

Les bactéries du complexe *St. bovis* / *St. equinus* (CSBSE) se sont révélées comme l'espèce dominante et l'acidifiant principal dans les fermentations spontanées du *fènè*. Près de 29 laits acidifiés traditionnels ont été collectés dans la périphérie de Bamako (Mali), au Kenya et en Somalie et investigé pour la présence des bactéries CSBSE et les

résistances aux antibiotiques. 95 *St. infantarius* subsp. *infantarius* et 26 *St. gallolyticus* subsp. *macedonicus* on été isolés et typés par PCR, RFLP et rep-PCR jusqu'au niveau des groupes des souches. 13 des 20 bactéries CSBSE sélectionnées et isolées des différents produits montraient une résistance contre la tétracycline et portaient les gènes résistants aux antibiotiques *tet*(M), *tet*(L) et *tet*(S). Cinq de ces souches montraient une multi-résistance contre ampicilline, chloramphénicol, clindamycine, érythromycine, gentamicine, néomycine, pénicilline, streptomycine et tétracycline et seulement cinq étaient sensibles à tous les onze antibiotiques testés. La résistance combiné d'érythromycine et de tétracycline de deux souches de *St. gallolyticus* subsp. *macedonicus* était basé aux gènes de résistance d'*erm*(B) et de *tet*(M).

118 entérocoques et 84 non-entérocoques BAL étaient testés sur la susceptibilité aux antibiotiques. La résistance contre la tétracycline était directement liée aux ventes d'oxytétracycline dans la saison précédante, avec 40%, 26% et 17% d'entérocoques résistantes à la tétracycline trouvées pendant les saisons froide, chaude et hivernale, respectivement. Les entérocoques résistantes à la tétracycline portaient les gènes de résistance *tet*(L), *tet*(M) et *tet*(S). La résistance contre la tétracycline était aussi dominante avec 17% parmi des non-*Enterococcus* BAL et était représenté par le gène *tet*(M) dans un *Lb. plantarum* et par le gène *tet*(S) dans quatre *Lc. lactis* subsp. *lactis* et sept *St. infantarius* subsp. *infantarius.*

Dix isolats parmi 1583 de la microflore du *fènè* correspondaient à quatre groupes de souches de la nouvelle espèce *Vagococcus teuberi* ssp. nov. *V. teuberi* qui était phénotypiquement résistante à la tétracycline (CMI (concentration minimal d'inhibitation) 64 µg/ml) et trimethoprim (CMI 256 µg/ml), mais portait seulement le gène de résistance trimethoprim *dfr*(G).

La sélection de 51 BAL sensibles aux antibiotiques d'après leur propriété d'acidification, activité antimicrobienne et leur production des exopolysaccharides a abouti au développent d'un ferment composé de deuxespèces consistué de *St. thermophilus* (un ferment industriel pour remplacer le *St. infantarius* subsp. *infantarius*) et *Lb. plantarum* ainsi que d'un ferment défini multi-espèces (FME) consistué de *St. thermophilus, Lb. plantarum, Lc. lactis* subsp. *lactis* et *W. paramesenteroides.* Les deux ferments montraient des activités antimicrobiennes élevées contre *Listeria invannovii* HPB28 et

Enterococcus faecalis DSM 2048^T à 30℃ et 35℃ et aussi un potentiel d'acidificati on élevé et une bonne stabilité pendant cinq cultures « back-slopping » à 35℃ et 40℃. Le FME et un ferment industriel de *St. thermophilus* étaient appliqués dans les fermentations du *fènè* à la mini-laiterie au Mali à température ambiante (27℃-36℃). L'acidification c'est déroulée plus rapidement pour le FME à un pH de 4.8±0.2 après 9 heures. La recontamination du lait chauffé et inoculé par le ferment par les pathogènes opportunistes était plus faible mais pas complétément éliminé comparativement à la fermentation traditionnelle du *féné.*

Conclusion:

La composition de la microflore de la fermentation spontanée du *fènè* dépend de la saison et de l'environnement de production et montre une biodiversité élevée des bactéries lactiques. Les pathogènes opportunistes sont dominants à côté des lactobacillies, lactocoques, pediocoques et *Weissella* dans la microflore du *fènè* et la bactérie principale de la fermentation spontanée du *fènè* est le pathogène pour les animaux et les hommes *St. infantarius* subsp. *infantarius.* La microflore du *fènè* contient un pool de gènes de résistances aux antibiotiques transférables. Les mêmes gènes de résistance à la tétracycline ont été trouvés parmi plusieurs genres. La recontamination du lait chauffé pendant la fermentation était réduite mais pas éliminée avec l'application de ferment. L'acidification rapide a été confirmé au laboratoire et à la mini-laiterie pour le nouveau ferment défini multi-espèces. La sécurité alimentaire et la qualité du *fènè* pourraient augmenter avec une fermentation standardisée et contrôlée en température, des mesures hygiéniques rigoureuses et l'interdiction d'utilisation d'antibiotiques non-contrôlés dans l'élevage.

En résumé, nous avons analysé pour la première fois les dynamiques de croissance et les fluctuations des souches dans la fermentation spontanement du *fènè,* caracterisé la nouvelle souche *V. teuberi* sp. nov., determiné un grand pool de gènes de résistance à la tétracycline et finalement developé un ferment sûr et adapté, dont l'application a été prouvée en production rurale au Mali.

1 Introduction

1.1 Mali: economy, demographic and its pastoral tradition

Mali lies landlocked in the middle of Sub-Saharan West Africa, the Sahel. The Republic of Mali formerly known as the Sudanese Republic and Senegal won independence from France in 1960 and, after a military coup in 1991 emerged as one of the strongest democracies in Africa. 36.1% of 12.3 million people in Mali live at or below poverty and 72% survive with less than 2 US$ per day (2007/2008). The per capita gross domestic product (GDP) is 1000 US$ with an estimated growth rate of 2.5% in 2007 (CIA 2008). Mali is ranked near the lowest position at 173 out of 177 on the human development index published by the UNDP, (2007/2008). Eighty per cent of the population works in agriculture contributing 35.7% to the GDP (FAO 2005). Small-scale traditional farming is the largest sector in low income agro-pastoral economy (FAO 2005).

Although Mali has well-established pastoral traditions with current estimates of 6.9 million cattle and 17.6 million small ruminants (FAO 2005), dairy imports exceed 15 billion Fcfa (ca. 39 million Swiss Francs) per year (DNS 1999). Over 70% of dairy imports into sub-Saharan countries are consumed in urban areas (Achuonjei and Debrah 1990) and Mali cannot satisfy its urban demand for dairy products (Achuonjei and Debrah 1990); between 71% and 86% of total expenditures on dairy foods were directly imported (Sissoko et al. 1990). Forty-five thousand out of an estimated 580’000 tons of milk consumed countrywide per year were imported (FAO 2005-2006) and, if Malians could afford they would drink more milk (Hetzel et al. 2004). This may be due to that milk is mostly produced for personal use and the combined impacts of desperate factors in local – often undocumented – trade; inefficient marketing, low production, poor collection systems, inappropriate pricing and market location in concert with abnormal trade margins set by numerous market intermediaries from producers to consumers (Achuonjei and Debrah 1990). Milk is the predominant protein source in the local diet accounting for 4.4% of the average daily caloric intake (FAO 2005-2006). Fermented milks such as *fènè* (see chapter 1.2.3) or lait caillé traditionally produced by women at home are very popular. Recent studies report that community-based milk processing at small-scale low-tech dairies increases product safety and contribute significantly to increased quality of life of local farmers (Bonfoh et al. 2005a). However, knowledge on

indigenous sour-milk production is transferred from household to small-scale production, without adapting basic hygienic measures needed for larger scale production.

1.2 Milk Fermentation

1.2.1 Historical Background

Spontaneous fermentation and preferential selection of those foodstuffs whose palatability was increased by this process, remains the origin of consumable fermented products (Stiles 1996) and allowed our ancestors keeping foodstuffs at ambient temperature. Fermented milk was first produced around 10'000 years ago (Tamime 2002) when the Neolithic Revolution transformed hunters and gatherers into settled land-based communities. The first evidence that cattle, sheep and goats were domesticated for milk production dates back to 5000 BC (Teuber 1995)[1]. Milk fermentation is dating to 1300 BC cited in the Old Testament (Roginski 1988). Cheese and butter consumption by the general population in the ancient Kingdom of Meroe (690 BC – AD 323) is recorded in detail (Abdelgadir et al. 1998). Repeated use of naturally-occurring microbiota to inoculate fresh milk, commonly referred to back-slopping, has been the developmental keystone of traditional fermented milk products worldwide (Wouters et al. 2002). Milk fermentation into specific products (e.g. yoghurt or cheese) was only possible because it provides unique growth conditions for lactic acid bacteria (LAB) (Teuber 1995). The specific role of individual microorganisms in fermentation was elucidated through pasteurization[2] studies lead by researchers in food preservation. These studies contributed directly to the defined integration of microorganisms in commercial food processing (Ross et al. 2002). An increased demand on food preservation during the Industrial Revolution forced food processing from kitchens and cottages into large-scale industries (Stiles 1996). Cultured butter was first produced in 1890 in Denmark by a defined LAB culture (Roginski 1988)[3].

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[1] Sumerians documented agricultural products in 3200 BC (Teuber 1995) and a limestone relief depicts the production of cultured butter 2900-2460 BC (Roginski 1988).

[2] Due to his familiarity with brewers yeast in beer fermentation, in 1857 Pasteur attributed lactic acid production to a cultured strain he identified as lactic yeast (Pasteur 1857). Subsequent development of a wide variety of starter cultures has led to improved fermentation performance and increased product quality (Ross et al. 2002).

[3] Subsequently starter cultures were made as liquid cultures produced in Germany and the United States and after World War II, freeze dried cultures for direct inoculation were developed (Teuber 1995).

Progress in the area of the development of defined starter cultures has been driven by an increased awareness of commercial food safety[4] and the search for novel flavors, textures or potential health benefits. These putative improvements may however gravely threaten long-term biodiversity through the loss of indigenous microbiota and strain diversity of traditional fermented foodstuffs (Wouters et al. 2002).

1.2.2 Development and safety of fermented dairy products in Africa

Traditional fermentation methods are still used to preserve food where refrigeration is not available (Motarjemi 2002) and Africa probably has largest variety of fermented foods (Holzapfel 1997). Soured-milks have provided a protein staple in the general population throughout the history of African Nations (Caplice and Fitzgerald 1999). These cultural practices, handed down from generation to generation, may be lost through impending socio-economic changes (Beukes et al. 2001). Furthermore, these foodstuffs have economic and social significance (Beukes et al. 2001). Dairy industries are less developed in countries where high quantities of milk are processed into indigenous products. Inadequate transportation, problems in animal breeding and feeding and lack of technical knowledge has restrained developments in many areas.

Milk fermentation is generally practiced in developing countries at home- or small-scale by spontaneous fermentation. Contaminants from the immediate environment (air, microbial bio-film of the vat, working materials, and producers) compete for nutrients in spontaneous fermentation processes, which are influenced by external factors such as weather (season, temperature), location and raw material. Milk produced locally within individual households or sold at markets are often of poor quality with e.g. up to 10^6 cfu/ml *Enterobacteriaceae* (Bonfoh et al. 2003b; Bonfoh et al. 2006; Bonfoh et al. 2003c; Fané et al. 2005; FAO 1990) due to insufficient knowledge in hygiene practices of producers, lack of veterinary control and general difficult climate conditions. Thus an FAO study (FAO 1990) concluded that for West Africa traditional milk products will be produced for a long time to come and sour-milk technology is the only mean of local disposition. Consequently traditional milk products should be regarded as hazard for transmitting zoonotic diseases, i.e. *Mycobacterium bovis* (Müller et al. 2008) or *Brucella*

[4] Bacteriocinogenic cultures applied as starter or protective cultures and/or their antimicrobial substances can be used as biopreservatives in food. The broad band bacteriocin Nisin, produced by *Lactococcus lactis*, is still the only bacteriocinogenic preservative approved by FDA (2001), whereas protective cultures are effectively used by industries (Miescher Schwenninger and Meile 2003).

sp. (Fané et al. 2005; Steinmann et al. 2006). Increased food safety can be achieved with industrial milk processing technologies adapted to rural small-scale production.

1.2.3 *Fènè* a traditional fermented milk of Mali

Raw milk is traditionally transformed into *fènè* through spontaneous fermentation in the calabash of herdsmen in the field of West Africa. *Fènè is* a firm, creamy and slightly-sour yoghurt-like product. These creamy and slightly-sour attributes are currently the consumers standard for a "good *fènè*". *Fènè* production often depends on unique recipes and is handed down from generation to generation. Therefore, many local variations of *fènè* exist. Summarized, *fènè* is produced from heated (above pasteurization temperature) or raw cow[5] milk by spontaneous fermentation[6] at ambient temperature. The "real" *fènè* is produced from the upper part of the milk (cream), second quality *fènè* is produced from creamed milk and standard *fènè* is produced from milk. Traditionally, men oversee animal husbandry, whereas women are responsible for milk processing and marketing. This traditional task sharing changes when milk is collected by men at small-scale dairies. The first small-scale dairies, transferring 200 – 1200 liters milk a day were introduced in rural villages in 2004 (Bonfoh et al. 2005a). *Fènè* is still produced in these dairies by spontaneous overnight fermentation in plastic vats covered by a mosquito net (Figure 1) at ambient temperatures. It is still not clear how spontaneous fermentation is induced at domestic and small-scale *fènè* production and how the microbiota is composed, especially at small-scale *fènè* production, where milk is heat treated to over 80℃ and subsequently transfor med overnight into a set-style yoghurt-like sour-milk by spontaneous fermentation[7].

[5] In Mali milk is produced by Zebu cows (*Bos taurus indicus*), N'Dama (*Bos taurus taurus*) and their crossbreeds either with local or with exotic breeds.
[6] Some families' adapted back-slopping or take always the same vat and fermentation place.
[7] without inoculation of a starter culture.

Figure 1 Production of *fènè* at the small-scale dairy in Kasséla.
1) Milk delivering by local producers, 2) Quality control of milk by the alcohol test, 3) Milk sieving, 4) Milk ready for pasteurization, 5) Milk pasteurization, 6) Checking pasteurization temperature (80-85℃), 7) Packaging of pasteurized milk, 8) Spo ntaneous overnight fermentation, 9) Packaging of *fènè*.

1.3 Starter culture

1.3.1 Lactic acid bacteria in fermented milk products

Lactic acid bacteria (LAB) are phylogenetically members of the *Clostridium-Bacillus* subdivision of Gram-positive eubacteria (Stackebrandt and Teuber 1988), are catalase-negative, lack the Krebs cycle, are not strictly anaerobic and have a low G+C content of less than 53% (Limsowtin et al. 2002). The evolution of the bacterial classification system divided the lactic cocci from streptococci into streptococci, enterococci and lactococci. New genera arose within the LAB e.g. the food-technology associated genera *Aerococcus*, *Carnobacterium, Enterococcus, Lactococcus, Lactobacilli, Enterococcus*, *Pediococcus, Streptococcus, Tetragenococcus*, *Vagococcus*, and *Weissella* (Figure 2)

(Axelsson 2004). Many other genera[8] compromising only a few species (http://www.bacterio.cict.fr/validgenericnames.html) are conform to the broad definition of LAB but are not related to food-technology. From the phylogenetic point of view bacteria of the genus *Bifidobacterium* although producing lactic acid should not be regarded as LAB due to their high G+C content; they are classified as *Actinobacteria* (Schleifer and Ludwig 1995). LAB used as starter cultures are grouped according their metabolism (homofermentative versus heterofermentative), temperature response (thermophilic versus mesophilic), salt tolerance (over 6.5% NaCl) and production of lactic acid stereoisomers (L(+), D(-) and its racemate) (Teuber 1995). Since LAB were commercially introduced in the early 1900s for industrial-scale milk fermentation, demands have increased tremendously for high active, stable and bacteriophage resistant starter cultures (Mäyrä-Mäkinen and Bigert 2004). Starter cultures affect flavor, texture and final product quality. Although novel starters are more and more designed and studied through genetic engineering (Cogan et al. 2007), traditional screening and typing methods can not be replaced when new microbiota are studied. Genomics research now starts to link probiotic functionality to genetic information. The commercial interest in probiotics was probably the driving force to sequence the genomes of 13 strains with pretended probiotic properties (Klaenhammer et al. 2008). Siezen and Bachmann (2008) cited 20 genome sequencing projects relevant to dairy fermentations. More than 700 bacterial genome sequencing projects have been realized whereof lactobacilli seem to be the most relevant bacteria among LAB with 15 genome sequencing projects (http://www.ebi.ac.uk/genomes/bacteria.html, state October 2008).

1.3.2 Typing of lactic acid bacteria from complex food matrices

Historically, bacterial classification was based on morphological observation and developed through phenotypic characterization and today microbial identification is a polyphasic approach combining molecular and phenotypic methods (Gillis et al. 2005). Bacterial typing strategies are not uniform and have to be well designed accordingly to the research objective and available laboratory equipment. Complete phenotypic characterization studies were still done in recent years (Mathara et al. 2004) but more and more these methodologies are replaced or completed with culture based or culture

[8] *Abiotrophia*, *Alloiococcus*, *Atopobacter*, *Catellicoccus* recently described by Lawson et al. (2006). *Desemzia*, *Dolosicoccus*, *Dolosigranulum*, *Eremococcus*, *Gemella*, *Globicatella*, *Granulicatella*, *Ignavigranum*, *Lactosphera* emeded discriped as *Trichococcus* (Liu et al. 2002), *Melissococcus* and *Paralactobacillus* (Limsowtin et al. 2002).

independent molecular methods (Obodai and Dodd 2006; Zamfir et al. 2006). The LAB of the microbiota of spontaneous *fènè* fermentation processes in our studies were identified as followed: (1) Sterile sample collection and cooled transport to the analyzing laboratory (LCV, Bamako, Mali), (2) Determination of bacterial counts of presumptive bacterial genera with serial dilution on semi-selective growth media and selection[9] of isolates for further experiments, (3) Application of a polyphasic approach combining the phenotypic methods Gram-staining, catalase activity and microscope observation with the molecular methods PCR, RFLP, ARDRA, rep-PCR, PFGE and 16S rRNA gene sequencing. Gram-positive and catalase-negative rods or cocci were subsequently typed

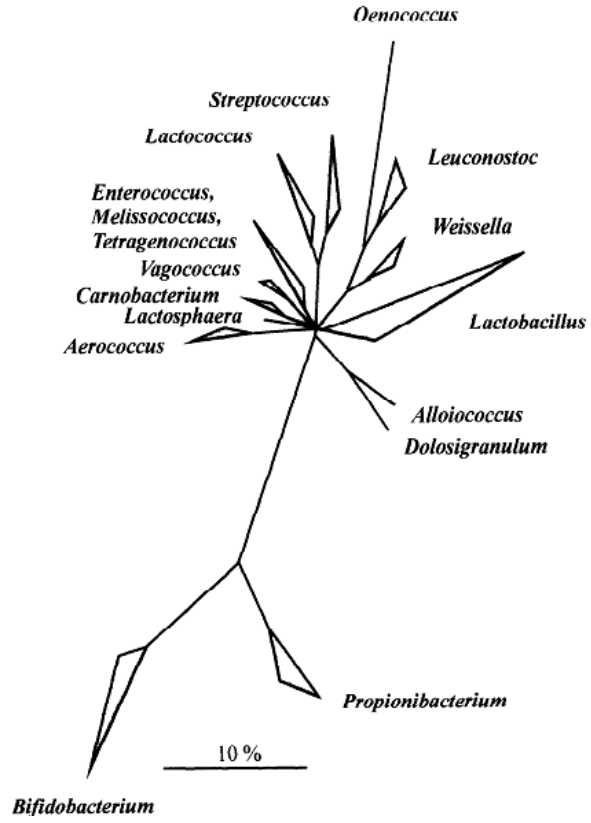

Figure 2 Phylogenetic groups of food related lactic acid bacteria (LAB), some genera conforming the broad definition of LAB but are not food related and bacteria with high mol% G+C in the DNA not belonging to LAB (Stiles and Holzapfel, 1997).

[9] Often a certain amount of colonies are randomly picked and pure streaked for further analysis based on defined selection criteria. High variety of LAB can be isolated with MRS and M17 media. Single genera or even species of LAB can be isolated with choosing more selected media conditions, such as differing pH of the media, incubation temperatures, carbon sources or antibiotic pressure. If a highly diverse micoflora should be isolated out of unknown sources the chose of less selective isolation condition might be appropriated.

at genus, species, subspecies and strain level with molecular methods. When novel isolates could not be assigned to a known species, they were classified as a new species following the *International Code of Nomeclature of Bacteria* (Sneath 2005)[10]. Well typed LAB might further be characterized on safety, technological and functional properties for developing new starter cultures.

1.3.3 Development of starter cultures for small-scale sour milk production

The microbiota of spontaneously fermented foodstuff evolved through back-slopping fermentations from domestic to industrial production (chapter 1.2.1). Starter cultures are separated either into defined or undefined cultures, whose microbiota composition are not documented or totally unidentified. Defined starters are applied as single- or mixed-strain starter cultures. Undefined starter cultures are used in spontaneous fermentation processes or with back-slopping techniques, which finally select for well adapted cultures (Wouters et al. 2002). Defined starters are applied as liquid or lyophilized cultures under well defined conditions in industrial countries. Adapted fermentation technologies have a large potential with regard to increased food safety and scalability of the process in developing countries (Motarjemi 2002). Adaption has to be realized with regard to logistic, electricity supply, infrastructure at rural small-scale production and training of people.

General criteria for developing new starters are safety measures, functional properties of the strains and consumer acceptance. In doing so, cultural habits of consumers have to be taken into account (Holzapfel 1997). Fast acidification of milk inhibits the growth of food born pathogens[11] and is therefore predominant for product safety. Starter cultures have to be resistant to bacteriophages for high yield industrial foodstuff production. Industry focuses on starters producing characteristic flavors, exopolysaccharide (EPS) or even bacteriocins. These attributes can nowadays be better investigated and improved

[10] General requirements to define new species are: (1) 16S rRNA sequence similarity of less than 97% between the closest relatives (Gevers et al. 2005) (2) The closest relatives sharing less than 70% of total genome DNA-DNA hybridization (Wayne et al. 1987) (3) And more than one strain of a new species have to be isolated (Stackebrandt et al. 2002). Whereas for the classification of unknown isolates at genus level no clear cut definitions are available (Gillis et al. 2005).

[11] Acidification below pH 4.5 inhibits the growth of pathogens such as *Enterobacteriacea* and *Listeria* (Khelef et al. 2006; Krämer 2002). *Staphylococcus aureus* does not grow below pH 4.0 (Krämer 2002). Product spoilage by contamination with moulds and yeast as well as enterococci and streptococci is still possible at lower pH.

by metabolic engineering (Teusink and Smid 2006). Sour milks are worldwide well accepted by consumers likely due to their distinct flavor. EPS production of bacteria leads to more consistent and creamy products and therefore, less solid compounds (milk proteins) have to be used for distinct organoleptic properties and higher production yields. Bacteriocin producing strains inhibit the growth of pathogens or spoilage microorganisms, permitting extended shelf life and replacing chemical ingredients, also known as E-numbers (Gálvez et al. 2008). Bacteriocinogenic strains or cultures are generally applied as protective strains or cultures and do not have to fulfill acidification or flavor properties like starters do.

Safety aspects of applied bacteria are generally based on the traditional use of LAB in various fermented food products. These LAB used in starter cultures have therefore the well known GRAS status (Generally Recognized as Safe) from the US Food and Drug Administration (FDA). The GRAS status is defined as any food additive approved as safe or substances used in food before 1958 (FDA 2001). The “Quality Presumption of Safety” (QPS) approach is a safety assessment of microorganisms throughout the food chain similar to the GRAS concept and is proposed to be included into the working program of the European Food Safety Agency (EFSA) (Chamba and Jamet 2008). In contrast to the GRAS concept the QPS approach assesses the threat of the presence of acquired antibiotic resistance factors of LAB applied in dairy products.

1.4 Antibiotic resistant bacteria in fermented food

The emerging risk of antibiotic resistant bacteria (ARB) has been highlighted by nosocomial infections with multi-resistant bacteria. Antibiotic resistance is cited as a shadow epidemic (Ammor et al. 2007) impacting human life quality more than global warming (Planta 2007). ARB from the environment i.e. food animals enter the food chain in high numbers (Aarestrup 2005; Aquilanti et al. 2007), which is alarming, for fermented ready to eat or probiotic food (D'Aimmo et al. 2007; Danielsen and Wind 2003; Kastner et al. 2006; Teuber et al. 1999). Bacteria harboring transferable antibiotic resistance genes can act as resistance gene reservoir in the gastrointestinal tract of humans and animals, where conjugative gene transfer is increased due to high bacteria concentration (Ammor et al. 2007; Kastner et al. 2006). The potential health hazard of these antibiotic resistant genes lies in their transferability between different genera (Aarestrup 2005; Perreten et al. 1997; Teuber et al. 1999), and resistance might be conferred to opportunistic pathogens (Aarestrup 2005; Okeke et al. 2005). The development of

microbial antibiotic resistances, a natural evolutionary process cannot be halted (Courvalin 2005; Levy 2001).

Paradoxically, antibiotic resistance in food-related bacteria has increased since drugs were introduced in animal husbandry therapy and prophylaxis. It has been shown that a reduction of antibiotic use leads to a decrease of ABR (Aarestrup 2005; Kaszanyitzky et al. 2007). These findings could not be confirmed for organic milk production in Switzerland, for which antibiotic treatments are very restricted, compared to ordinary milk production (Roesch et al. 2006; Walther and Perreten 2007). Antibiotic use in low-scale agriculture of developing countries is small compared to industrialized countries and only applied for therapeutic measures. In Mali, as in other developing countries, no governmental regulations on the use of antibiotics in agriculture are existent (Levy and Marshall 2004; Okeke et al. 2005). Therefore, antibiotics are available without prescription and are applied inappropriately in sub-therapeutic doses for animal husbandry, favoring antibiotic resistance formation in bacteria present in the agro-food chain (Aarestrup 2005; Manie et al. 1999; Rahim et al. 2007) and antibiotic residues in food (Bonfoh et al. 2003a). Antibiotics usage should be very tightly restricted and banned as a growth promoter in animal husbandry globally to reduce the emerging risk of ABR in the agro-food chain worldwide. Summarized it has been shown that ABR enter the food chain and are found in high numbers in fermented foodstuff. However, to our knowledge little is known about the presence of ABR in spontaneously fermented milks of Africa although it is known that uncontrolled antibiotic use favors ABR emergence.

1.5 Objectives of this study

1.5.1 Background

Mali is a traditional agro-pastoral country, with small-scale farming practiced in large part (FAO 2005)[12]. The cornerstone for advanced scientific work in the Malian dairy and husbandry sector, leading to the development of the local market and reduction of poverty, was laid with the start of the project "Healthy milk for the Sahel" in the year 2000 (Alfaroukh and Djiteye 2003). The project "Healthy milk for the Sahel" aims to improve milk quality in the Sahel and is composed into the three branches: animal health, sociological influences and milk processing. Animal health is crucial for farmer's income and long-term development of the local dairy market[13]. Several studies analyzed the impact of the current Malian dairy market on public health (Bonfoh et al. 2002; Fané et al. 2005; Hetzel et al. 2004; Steinmann et al. 2005; Steinmann et al. 2006), private earnings (Bonfoh et al. 2005a; Sall 2002) and consumer behavior (Aarestrup 2005; Hetzel et al. 2005). Milk quality (Bonfoh et al. 2003b; Bonfoh et al. 2003c; Bonfoh et al. 2005b) and antibiotic residues in milk (Aarestrup 2005; Bonfoh et al. 2003a) were well analyzed before hygiene measures were adapted (Bonfoh et al. 2006) and finally, small-scale dairies were successfully introduced by the Vétérinaires Sans Frontière Suisse (Bonfoh et al. 2005a). Traditionally, people consumed milk raw or spontaneously fermented by environmental strains. Milk often could not be heat treated due to a high load of contaminating bacteria acidifying milk and subsequently proteins precipitating during heating (Bonfoh et al. 2003b; Bonfoh et al. 2003c). Furthermore, a high prevalence of *Brucella abortus* in milk and of tuberculosis in animals (Bonfoh et al. 2002; Fané et al. 2005; Müller et al. 2008) both zoonotic infections, related the consumption of milk to public health hazards. Public health hazard could be mitigated with adequate milk heating and standardized milk fermentation. Therefore, studies on the spontaneous fermentation process were requested with the objective to develop an adapted starter culture for standardized *fènè* fermentation at small-scale production.

[12] Deficiency (chapter 1.1 and 1.2.2) in the development of the local Malian dairy market was reported (Achuonjei and Debrah 1990; Sissoko et al. 1990).

[13] Therefore, reasons for calf mortality were well studied in the frame of the PhD of Monica Natalie Wymann (Wymann et al. 2006; Wymann et al. 2007; Wymann et al. 2008) and let to substantial recommendations for future research, national authorities and livestock producers (Wymann 2005).

1.5.2 Hypotheses

Five hypotheses were formulated for this thesis:

1. Environmental microbiota induces spontaneous *fènè* fermentation, in which lactic acid bacteria (LAB) are of predominant importance.
2. The microbial biodiversity of spontaneous *fènè* fermentation processes depend on seasons.
3. Uncontrolled antibiotic use in animal husbandry favors antibiotic resistance formation in LAB.
4. The potential health hazard to consumers of *fènè* is reduced with adaption of a new developed starter culture.
5. Investigations of unknown microbiological sources lead to the isolation of new species.

1.5.3 General objectives

The general objectives of this work were:

1. To investigate the microbial ecological diversity of raw and heated milk *fènè* fermentation processes at home- and small-sale during cold, hot and rainy seasons.
2. To elucidate potential health hazards to consumers of *fènè*.
3. To develop an adapted starter culture for local small-scale *fènè* production.

1.5.4 Specific objectives

- To characterize spontaneous *fènè* fermentation processes of heated milk in small-scale as well as raw and heated milk in domestic production during cold, hot and rainy seasons on bacterial counts, pH and temperature profiles.
- To isolate LAB from these spontaneous *fènè* fermentation processes, type isolated bacteria to strain level using a suitable molecular approach to type large numbers of LAB and analyze environmental effects on strain diversity
- To investigate health hazards of spontaneous *fènè* fermentation processes relating to the occurrence of human and animal pathogens and antibiotic resistances.
- To characterize selected LAB on acidification properties, antimicrobial activity and EPS production and develop and evaluate mixtures of defined starter cultures by successive back-slopping cultures.
- To field test defined starter cultures at small-scale level and conduct preliminary sensorial consumer tests.

2 Quantitative identification and seasonal fluctuations of microbial diversity in *fènè*, a spontaneously fermented Malian sour milk

Data presented in this chapter are submitted for publication to an international peer-reviewed journal in 2009 in a slightly changed version with the authorship: Stephan Wullschleger, Christophe Lacroix, Bassirou Bonfoh, Aminata Sissoko-Thiam, Selina Baltzer, Edwina Romanens, Sarah Baumgartner, Idrissa Traoré, Marcus Yaffee and Leo Meile

2.1 Abstract

A simple and powerful strategy for rapid, unequivocal identification/quantification of individual bacterial strains is applied through the combined investigation of microbiological diversity and seasonal variation of microbial populations in the spontaneously fermented Malian sourmilk, *fènè*, a traditionally important dietary protein source of rural and urban Malians. Native *fènè* microbiota was assayed during three seasons (hot, cold and rainy) from single household and a small-scale dairy. Microbial traps mapped in parallel with production equipment swabs provided initial coordinates tracking bacterial contaminant's point of entry into small-scale heat-treated milk *fènè* production. A total of 1583 catalase-negative, Gram-positive bacteria were isolated from 98 samples. Lactic acid bacteria (LAB) were initially isolated on semi-selective media i.e., presumptive lactobacilli on Man Rogosa and Sharpe (MRS), lactococci on M17 and enterococci on Azide maltose (KF) agar. Lactobacilli, lactococci and enterococci populations fluctuated seasonally with minima of 7.9±0.5, 8.2±0.8 and 7.8±1.0 Log_{10}(cfu/ml) and maxima of 9.2±0.5, 9.2±0.3 and 8.8±0.3 Log_{10}(cfu/ml) from rainy to hot season, respectively, with high species biodiversity levels identified [isolates/strain]: *Lactobacillus fermentum* (77/18), *Weissella confusa* (75/21), *Lactococcus lactis* subsp. *lactis* (62/21), *Pediococcus pentosaceus* (36/30), *Lactobacillus plantarum* (24/9), *Weissella paramesenteroides* (11/3), *Lactococcus garvieae* (11/0), *Lactobacillus* sp. (10/0) and *Pediococcus acidilactici* (8/4). Variation of microbial strain biodiversity was measured by seasonal strain population fluctuations with respect to each production environment.

Presumptive *Enterobacteriaceae* isolated on Violet Red Bile Glucose (VRBG) (6.5 - 9.0 Log_{10}(cfu/ml)) and *Staphylococcus aureus* isolated on Baird Parker (BP) (5.2±0.9 Log_{10}(cfu/ml) indicated an elevated health hazard to consumers. This was further corroborated with the identification of 596 enterococci and 454 streptococci from 1583 isolates as dominant populations of *fènè* microbiota. The conspicuous absence of *Streptococcus thermophilus*, usually occupying a central fermentative role, was further underscored by the finding that 87 streptococci selected for further typing were all found to be the human and animal pathogen *St. infantarius* subsp. *infantarius*. These findings clearly identify spontaneously fermented *fènè* as a reservoir for the pathogen *St. infantarius* subsp. *infantarius,* and as a likely source for human infection transmission.

2.2 Keywords

Fènè; spontaneous milk fermentation; lactic acid bacteria; *Streptococcus bovis*; *St. infantarius* subsp. *infantarius*; PCR; seasonal influence; strain diversity; ecological biodiversity; milk; Africa; Mali

2.3 Introduction

Fermented milk *(fènè)* is a principle protein source for people living in rural Mali where ancient pastoral traditions live on and spontaneous fermentation is the sole option for preserving milk. *Fènè* is typically sour (pH between 4.1 and 4.9) with a homogenous creamy consistency and consumed either as a yoghurt-like beverage or used in sauces like fresh or sour cream. Malians would certainly consume more dairy products if they could afford it (Hetzel et al. 2004). However, an unacceptably high level of putative human microbial pathogens is unfortunately found in milk sold at public markets (Bonfoh et al. 2003c).

Lactic acid bacteria (LAB) play a central fermentative role among variable populations of other microbial contaminants transmitted from the immediate surroundings to spontaneously souring milk during production of traditionally fermented milks of Africa. Historically, phenotypic as well as culture-independent and -dependent molecular methods have been used to type bacteria at species level in spontaneously fermented African milks (Isono et al., 1994; Mathara et al., 2004; Obodai and Dodd, 2006). The central fermentative role of LAB has been established throughout a wide range of traditional African fermented milk products with lactobacilli, lactococci and enterococci as the predominant genera among LAB (Abdelgadir et al. 2001; Benkerroum and Tamime 2004; Beukes et al. 2001; Chammas et al. 2006; Gonfa et al. 2001; Gran et al. 2002; Hamama and Bayi 1991; Isono et al. 1994; Kebede et al. 2007; Mathara et al. 2004; Miyamoto et al. 2005; Mutukumira 1995; Narvhus and Gadaga 2003; Obodai and Dodd 2006; Olasupo et al. 2001), whereas *St. bovis* group has rarely been identified (Abdelgadir et al. 2008; Gonfa et al. 2001; Isono et al. 1994).

This study is the first comprehensive on-site study analyzing seasonal and production-driven microbial diversification – tracking growth dynamics of single strains unequivocally identified in situ through molecular typing. We focused in this study on the unequivocal typing of bacteria belonging to the “healthy” fermentative microbiota rather than enterococci or streptococci, which were mostly only typed to genus level.

2.4 Materials and methods

2.4.1 Sample collection

Small-scale dairy and domestic *fènè* production were investigated in Mali during the hot (February and March 2005), cold (November and December 2005) and rainy (June and July 2006) seasons. The Bagan Yiri Wa Ton communal dairy cooperative and domestic production site [family farm] are both located in Kasséla, a village 40 km from the capital Bamako. *Fènè* fermentation processes were sampled and processed twice per season at seven days intervals. Domestic samples of raw milk and milk heated during 30 minutes from ambient temperature (30.4±6.4℃) to 85 ℃ – 90℃ were prepared in three liter batches at the family farm for domestic production. Sampling (Table 1) from domestic production was organized as followed: Calves were permitted to suckle prior to hand-milking and farm milk was extracted manually from a single teat (samples L1) directly into a sterile 100 ml glass bottle or gathered from the collective milk basin (Lm) of the 10 – 15 head cow dairy herd. Milk was then heated in a metal kettle, cooled for 30 min to 45℃ – 50℃ in open plastic or metallic vats at ambient temperatures for *fènè* production from heated milk. Unheated milk was poured into another plastic vat for raw milk *fènè* production. Both vats were covered with a ceramic or metallic plate and placed in the family's hut for spontaneous overnight fermentation. Samples (Table 1) of heated and raw milks (F0), and fermented milks after 2, 4 h (F2 and F4) and after approximately 22 h fermentation (Ff) were collected, split into two equivalent aliquots of 50 ml into sterilized 100 ml glass bottles, stored and transported on ice at 4 – 8℃ and analyzed at the Laboratoire Central Vétérinaire, Bamako. Milk and ambient temperatures as well as pH (mobile pH-meter 604, Metrohm AG, Herisau, Switzerland) were recorded immediately after sample collection. *Fènè* production and sampling (Table 1) at small-scale production was slightly different than at domestic production. The communal pool of raw milk (ca. 50 farmers) was heated during 50 minutes from ambient temperature (30.6±4.2℃) to 85℃ – 90℃ and twenty liters aliqu ots were transferred into 30 liters plastic vats then covered with a mosquito net, permitted to cool to 45℃ at ambient temperatures and spontaneously fermented overnight on site. Samples were taken and treated as indicated for domestic production (F0, F2, F4 and Ff), additional samples were taken after 8 h (F8) and 18 h (F18) of spontaneous fermentation.

Table 1 Sampling schedule of spontaneous *fènè* fermentation processes at domestic and small-scale production repeated twice per season

Period	Fermentation process		Sampling intervals [a]
Hot season	domestic	raw milk	L1, Lm, F0, F2, F4, Ff
		heated milk	F0, F2, F4, Ff
	small-scale	heated milk	Lm, F0, F2, F4, Ff
Cold season	domestic	raw milk	L1, Lm, F0, F2, F4, Ff
		heated milk	F0, F2, F4, Ff
	small-scale	heated milk	Lm, F0, F2, F4, F8, F18, Ff
Rainy season	domestic	raw milk	L1, Lm, F0, F2, F4, Ff
		heated milk	F0, F2, F4, Ff
	small-scale	heated milk	Lm, F0, F2, F4, F8, F18, Ff

[a] Abbreviations of the sampling points: L1: milk from one teat of the cow's udder; Lm: pooled milk; F0: start point of the spontaneous fermentation; F2, F4, F8, F18: 2h, 4h, 8h, 18h after fermentation started; Ff: final product (approximately 22 h after fermentation started).

2.4.2 Microbiological analysis

The production environment and equipment of the small-scale dairy were assayed using microbiological traps, i.e. 9.4 cm diameter open plastic Petri dishes filled with Man Rogosa and Sharpe (MRS) (pH 6.4) (Labo-Life Sàrl, Pully, Switzerland), M17 (Labo-Life Sàrl) and Azide Maltose (KF) (Labo-Life Sàrl) agar were placed for one hour on the mosquito net and on a container one meter from the fermentation vat. Further samples were taken with a sterile cotton swab from the fermentation vat surface and streaked on MRS (pH 6.4), M17 and KF agar plates. Bacterial counts of milk samples were determined through serial dilution (10^{-1} – 10^{-8}) in sterilized peptone solution [0.85% (w/v) NaCl and 0.1% (w/v) peptone (VWR-International, Dietikon, Switzerland)]. 0.1 ml aliquots were spread in duplicate on selective media; Plate Count agar (PC) (Becton Dickinson, Allschwil, Switzerland) for aerobic mesophilic counting, MRS agar (pH 6.4) for presumptive lactobacilli, M17 agar for presumptive lactococci, KF agar for enterococci, Violet Red Bile Dextrose agar (VRBD) (VWR-International) for *Enterobacteriaceae* and Baird Parker agar (PB) (Labo-Life Sàrl) for presumptive *Staphylococcus aureus* (in the hot season). MRS plates were incubated anaerobically at 37℃ for 48 h using anaerobic jars with an atmosphere generation system (AnaeroGen, Oxoid AG, Basel, Switzerland). PC and M17 plates were incubated at 30℃ for 24 h, KF plates at 43℃ for 48 h and PB and VRBG plates at 37℃ for 24 h aerobic. A minimum of five colonies per dilution and

media were picked from MRS, M17 and KF agar plates based on color, shape and colony size differences and purified by streak plating at least twice on the same medium. Isolates were phenotypically pre-typed by Gram-staining (Gregersen 1978), catalase activity testing (3% H_2O_2) and microscopic observation. MRS, M17 and KF isolates were sub-cultured in 2 ml Cryo-Vials (Huber + Co, Reinach, Switzerland) containing 1 ml MRS, M17 and Brain Heart Infusion (Becton Dickinson), respectively for 24 h. Cultures were then transported within 24 h at ambient temperature to our laboratory at ETH Zurich, mixed with 0.7 ml sterile glycerol (98%) and stored at – 80°C.

2.4.3 Bacterial DNA extraction and molecular typing strategy to strain level

Relative numbers of typed genera and species were determined based on MRS counts of isolates grown on [unacidified] MRS agar plates (pH 6.4). DNA of single colonies of catalase-negative Gram-positive isolates was isolated as previously described (Goldenberger et al. 1995). 1.5 µl of this DNA template was used in PCR. Reference strains used in typing experiments are listed in Table 2.

Morphological and phenotypic tests and molecular methods were combined in a flow-chart protocol (Figure 3) facilitating rapid typing of large numbers of putative LAB. PCR amplification was performed in a Biometra®TGradient Cycler (BioLabo, Châtel-St.Denis, Switzerland). Reaction mixtures, amplification programs, oligonucleotide primers and references are displayed in (Table 3). Coccoid presumptive LAB were first typed with an enterococci-specific PCR (Ke et al. 1999) and bacilli with the lactobacilli-specific PCR (Dubernet et al. 2002). Coccoid LAB that did not display a positive signal for enterococci were subsequently typed with lactococci genus- and species-specific PCR/RFLP (Deasy et al. 2000; Pu et al. 2002). Presumptive coccoid LAB displaying negative signals in both the enterococci- and lactococci-specific PCR were investigated with a *St. thermophilus*-specific (Lick et al. 1996) and a streptococci genus-specific PCR (Picard et al. 2004). Lactococci had to be typed prior to streptococci because Picard et al., (2004) reported positive signals for *Lc. lactis* subsp. in the streptococci-genus specific PCR. Presumptive coccoid LAB, outside of the genera enterococci, streptococci or lactococci were investigated with pediococci-specific typing (Mora et al. 1997). Those microbes testing consistently negative were subsequently tested with bacilli testing negative in the lactobacilli PCR (Dubernet et al. 2002) against *Weissella* species (Jang et al. 2002). LAB typed as lactobacilli were further typed at species level with a multiplex-PCR approach (Song et al. 2000). Bacteria belonging to the *Lb. plantarum* group are reported as *Lb.*

Table 2 Reference stains used for molecular typing experiments

Bacterial strains
E. faecalis DSM[a] 20478[T]
Lb. acidophilus DSM 20079[T]
Lb. casei ATCC[b] 334
Lb. delbrueckii subsp. *lactis* FBC[c] FAM-10991
Lb. delbrueckii subsp. *bulgaricus* DSM 20081[T]
Lb. fermentum DSM 20052[T]
Lb. plantarum DSM 20174
Lb. salivarius FBC DC 6
Lb. reuteri FBC SD 2112
Lc. garvieae DSM 20684[T]
Lc. lactis subsp. *lactis* DSM 20481[T]
Lc. lactis subsp. *cremoris* DSM 20069[T]
Lc. raffinolactis DSM 20443[T]
P. acidilactici DSM 20284[T]
P. pentosaceus DSM 20336[T]
St. bovis DSM 20480[T]
St. infantarius subsp. *infantarius* CCUG[d] 43820[T]
St. infantarius subsp. *coli* CCUG 43822[T]
St. alactolyticus DSM 20782[T]
St. gallolyticus subsp. *gallolyticus* DSM 16831[T]
St. gallolyticus subsp. *macedonicus* DSM 15879[T]
St. thermophilus DSM 20617[T]
W. confusa DSM 20196[T]
W. paramesenteroides DSM 20288[T]

Abbreviations of the genera: *E. = Enterococcus; Lb. = Lactobacillus; Lc. = Lactococcus; P. = Pediococcus; St. = Streptococcus; W. = Weissella.*

[a]DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig Germany.

[c]FBC, Collection of the Laboratory of Food Biotechnology, ETH Zurich, Switzerland.

[d]CCUG, Culture Collection, University of Göteborg, Sweden.

plantarum and not further investigated within this study. Species of lactobacilli, *Weissella*, pediococci and *Lc. lactis* subsp. *lactis* were typed into strain clusters by rep-PCR. 18 isolates identified as streptococci were typed with 16S rRNA gene sequencing (Microsynth, Balgach, Switzerland) to species level and used to incorporate an additional typing protocol (Chen et al. 2008) to the flow-chart (Figure 3).

16S rRNA gene sequences were analyzed with NCBI data base matching (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared on base pair homology (http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi).

2.4.4 Typing at strain level

Pulsed-field gel electrophoresis (PFGE) represents the “golden standard” for molecular typing at strain level (Olive and Bean 1999). The repetitive extragenic palindromic (Rep) elements PCR exhibits a slighter discriminatory power but still a good correlation with PFGE results and is applied to type large numbers of isolates (Olive and Bean 1999). We therefore compared these techniques on accuracy and throughput capacity with 37 isolates (*Lactobacillus fermentum* (24), *Lb. plantarum* (3), *Streptococcus infantarius* subsp. *infantarius* (7) and *Lactococcus lactis* subsp. *lactis* (3)) from a first batch of samples from the hot season.

Isolates were grown to an optical density (OD_{600}) of approximately 6.0±0.5 for lactobacilli, 2.4±0.3 for *St. infantarius* subsp. *infantarius* and 0.6±0.1 for *Lc. lactis* subsp. *lactis* for PFGE experiments. DNA plugs were processed according to Simpson et al. (2002). DNA isolated from *St. infantarius* subsp. *infantarius* (Bouton et al. 1998), lactobacilli (Coeuret et al. 2003) and *Lc. lactis* subsp. *lactis* (Tanskanen et al. 1990) was digested with *Sma*I, *Not*I and *Sma*I, respectively.

A modified rep-PCR was adapted from the protocol of Kostinek et al. (2005) using the $(GTG)_5$ primer (Gevers et al. 2001). Amplification was carried out in a Biometra®TGradient Cycler (BioLabo) with an initial denaturation step at 95℃ for 5 min, followed by 30 cycles with denaturation at 95℃ for 30 sec, annealing at 40℃ for 1 min, extension at 62℃ for 3 min and a 8 min final exten sion step at 65℃.

PCR and PFGE gels were stained with ethidium bromide and visualized with a UV transilluminator. Gel images were captured using the AlphaImager™ system (Alpha Innotech Corporation, San Leandro, California, USA). The digitized images were normalized to a TriDye 100-bp and 1-kb DNA ladder (BioConcept, Allschwil, Switzerland). PFGE and rep-PCR fingerprints were grouped with the unweighted pair-group method using arithmetic averages clustering algorithm (UPGMA) (Sneath and Sokal 1973). Dendrograms were constructed with the program http://www.tinet.org/~debb/UPGMA (Garcia-Vallve et al. 2000).

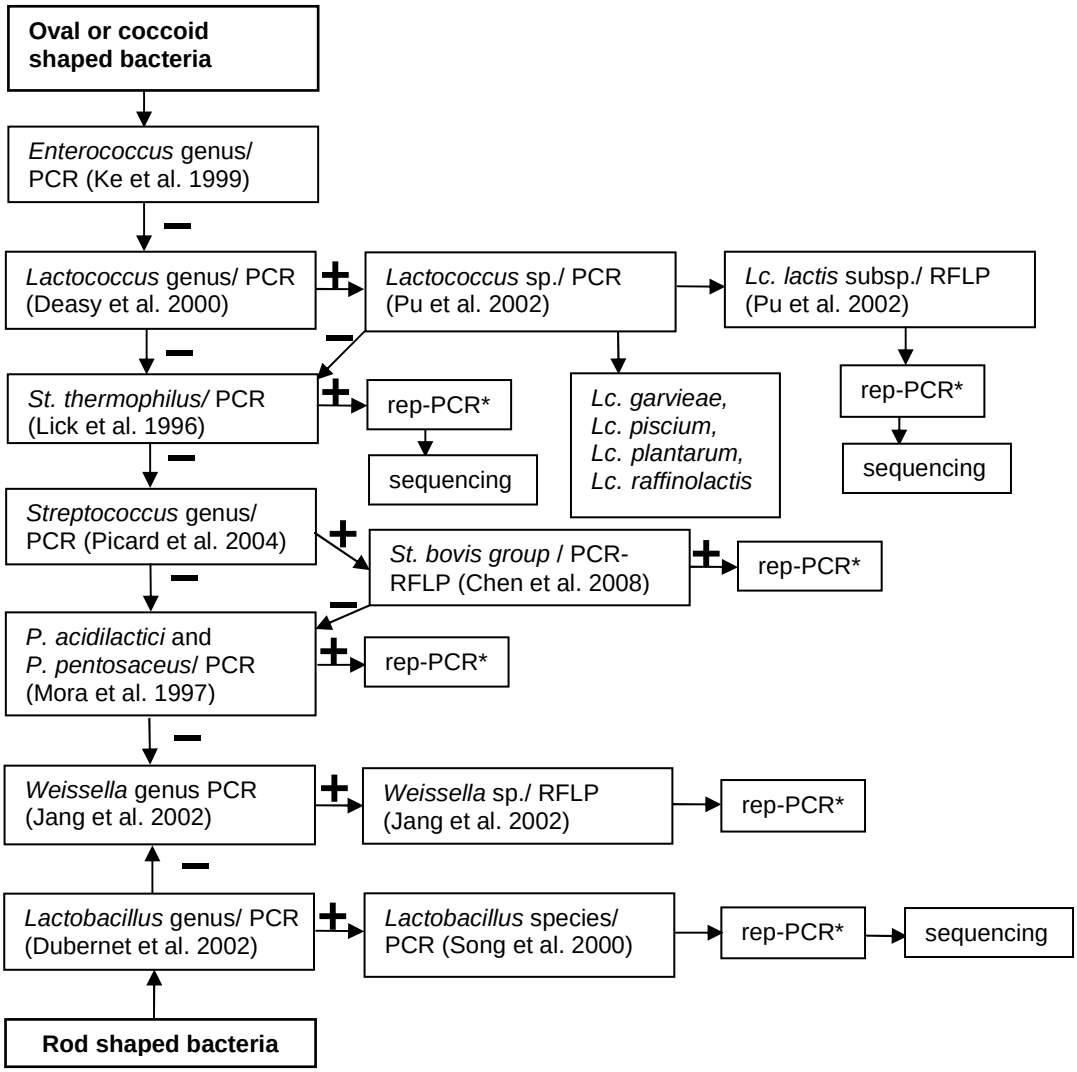

Figure 3 Flow chart of molecular approaches used for typing large numbers of presumable lactic acid bacteria. +: positive PCR result; -: negative PCR result. * modified from (Gevers et al. 2001; Kostinek et al. 2005).

2.4.5 Statistical analysis

Bacterial counts were normalized by Log_{10} transformation. Differences between means of bacterial counts [analyzed between seasons and production-type], pH and temperature were tested for statistical significance (p=0.05) using the Tukey's-b test of SPSS (ver. 16.0 SPSS, Chicago, IL, USA).

Table 3 PCR conditionsc used for typing bacterial isolates from *fènè* production at genus and species level

PCR target	$MgCl_2$ [mM]	Primer [uM][a]	Taq Polymerase [µl] [b]	Reaction volume [µl]	Annealing conditions [℃/sec]	Polymersation time [sec] at 72℃	Cycles	Heating rate [℃/sec]	Reference
Genus *Lactobacillus*	2.5	1	2.5	20	55/30	30	20	4	Dubernet et al. (2002)
Lactobacillus sp. groups	1.5	1	2.5	20	55/120	120	35	1.5	Song et al. (2000)
Lactobacillus sp. group III	1.5	1	2.5	20	62/120	120	35	1.5	Song et al. (2000)
*Lactobacillu*s sp. group IV	1.5	1	2.5	20	60/120	120	35	1.5	Song et al. (2000)
Genus *Lactococcus*	5	1	1.25	25	55/60	60	28	1.5	Deasy et al.(2000)
Species *Lactococcus*	1.5	1	1	50	45/30	60	35	1.5	Pu et al. (2002)
Genus *Enterococcus*	3	1	1.25	25	55/30	60	40	4	Ke et al. (1999)
Genus *Streptococcu*s	2.5	0.4	0.5	20	58/30	60	40	1.5	Picard et al. (2004)
St. thermophilus	1.5	1	0.5	20	50/70	30	30	1.5	Lick et al. (1996)
Genus *Weissella*	1.5	0.5	2.5	50	55/30	60	30	4	Jang et al. (2002)
Pediococcus acidilactici and *P. pentosaceus*	1.5	1	1.25	25	58/30	60	34	4	Mora et al. (1997)
16S rRNA gene universel	3	1	1.5	50	60/30	120	40	4	(Goldenberger et al. 1997; Greisen et al. 1994)

[a] Microsynth AG, Balgach, Switzerland.

[b] Sysmex Digitana AG, Horgen, Switzerland.

[c] Each mastermix contained the appropriate amount of 1x reaction buffer without $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate (GE Healthcare Europe GmbH, Otelfingen, Switzerland) and 2% Tween 20 (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) (Goldenberger et al. 1995). Each PCR was started with an initial denaturation at 95℃ for 5 min and finished with a polymer ization step at 72℃ for 7 min. Cycles were started with a denaturation step at 95℃ for 30 sec .

2.5 Results

2.5.1 Bacterial composition and pH of *fènè*

Significant seasonal fluctuations in bacterial counts observed in *fènè,* produced in duplicate by three different spontaneous overnight (22 h) fermentation processes from domestic and small-scale production sites during hot, cold and rainy seasons are reported in Table 4.

Highest bacterial counts of total aerobic mesophilic bacteria on PC-agar (9.4±0.2 Log_{10}(cfu/ml)), counts of presumptive lactobacilli on MRS (9.2±0.5 Log_{10}(cfu/ml)) and lactococci on M17 (9.2±0.3 Log_{10}(cfu/ml)) were measured during the hot season after spontaneous overnight (22 h) fermentation. Bacterial counts of presumptive enterococci (KF-agar) and *Enterobacteriaceae* (VRBG-agar) with 8.8±0.3 Log_{10}(cfu/ml) and 7.5±0.5 Log_{10}(cfu/ml) respectively, were very high during the year and all types of production. Counts for presumptive *Staphylococcus aureus* (BP-agar) reached 5.2±0.9 Log_{10}(cfu/ml) during hot season. Bacterial counts on PC-agar, MRS, M17 and KF plates were between 8.6±0.4 and 9.0±0.5 Log_{10}(cfu/ml) during cold season. Bacterial counts of these four media were significantly ($p<0.05$) lower during rainy season (7.8±1.0 – 8.4±0.6 Log_{10}(cfu/ml)) than during hot season (8.8±0.3 – 9.4±0.2 Log_{10}(cfu/ml)), but not from counts measured during cold season, which were intermediate. VRBG-counts were not significantly different between the seasons (7.5±0.6 – 7.9±0.7 Log_{10}(cfu/ml)).

The average ambient temperatures and pH remain seasonally constant (Table 4). The average milk temperature of 34.8±5.9℃ during domes tic fermentation of *fènè* produced from heated milk was significantly higher than during raw milk *fènè* production (Table 4). Production-type did not influence pH and bacterial populations, except VRBG counts. VRBG-counts at small-scale dairy production were significantly lowest with 7.1±0.4 Log_{10}(cfu/ml) (Table 4).

2.5.2 Microbiological analysis during spontaneous *fènè* fermentation processes

Mean bacterial counts from spontaneous *fènè* fermentation processes are presented in Table 5. Initial cell counts of pooled raw milk (Lm) used in domestic *fènè* production were significantly lower during cold and rainy seasons than during hot season ($p<0.001$), and also lower than at small-scale production ($p<0.002$). No seasonal effect on cell counts

was detected for raw milk (Lm) at small-scale production. Bacterial counts of domestic raw milk *fènè* production were significantly higher than those for heated milk *fènè* production at both domestic and small-scale production in the first four hours (F0, F2, F4) of spontaneous fermentations during hot and cold seasons, but not during rainy season and absolutely highest during hot season. Bacterial counts of heated milk *fènè* production at both domestic and small-scale during the first four hours (F0, F2, F4) of spontaneous fermentation processes were not influenced by seasons. Bacterial counts in *fènè* (Ff) produced from heated milk were higher in domestic production as in small-scale dairy production during cold and rainy seasons, but not during hot season.

The fate of the microbiota during spontaneous *fènè* fermentation processes is illustrated in Figure 4 by using one fermentation process per production-type during the cold season. Bacterial counts increased throughout fermentation of domestic raw milk *fènè* production and were reduced after milk heating in *fènè* production from heated milk (Figure 4, graphics A1, B1, C1). Bacteria colonizing heated milk were detected first on M17 and PC agar plates (F0), whereas no colonies were found on KF agar plates until the fourth hour (F4) of fermentation (Figure 4, A1, B1, C1).

Streptococci and to a smaller extent enterococci dominated spontaneous *fènè* fermentation (Figure 4, A2, B2, C2). Enterococci were detected in three samples (Ff, domestic heated milk fermentation and F4 and Ff small-scale heated milk fermentation) not on MRS agar plates but only on KF agar plates (results not displayed). No enterococci were detected in sample F2 during domestic heated milk fermentation.

W. confusa was present at 28% and 13% in the samples Lm and F0, respectively, *Lb. fermentum* between 15% and 33% in the samples F0 to Ff (results not displayed) and *Lc. lactis* subsp. *lactis* at up to 20% (sample F2) but only detected on M17 agar in the final product (Ff) (results not displayed) during domestic raw-milk *fènè* fermentation (Figure 4, A2).

W. confusa was detected at 9% in raw milk, whereas both *W. confusa* and *Lb. fermentum* were present with 5% to 33% in the samples F4 and Ff in domestic heated milk fermentation. *Lc. lactis* subsp. *lactis* was not found during this fermentation (Figure 4, B2).

Table 4 Bacterial counts on non-selective and selective agar media and pH of six *fènè* samples as well as ambient and milk temperatures of corresponding fermentation processes per season and type of production

Analysis within the season	pH	Ambient T℃*	Milk T℃*	Bacterial counts [Log $_{10}$ cfu/ml] on:				
				MRS	M17	KF	PC	VRBG
Hot season	4.4±0.2	31.5±5.3	33.9±3.4	9.2±0.5^{a,Y,X}	9.2±0.3^{a,Y,X}	8.8±0.3^{a,X}	9.4±0.2^{a,Y}	7.5±0.5^{a,Z}
Cold season	4.6±0.3	30.1±4.0	33.6±6.0	8.9±0.5^{a,Y}	9.0±0.4^{a,Y}	8.6±0.4^{a,b,Y}	9.0±0.5^{a,b,Y}	7.7±0.7^{a,Z}
Rainy season	4.8±0.7	30.2±6.5	33.5±5.3	7.9±0.5^{b,Z}	8.2±0.8^{b,Z}	7.8±1.0^{b,Z}	8.4±0.6^{b,Z}	7.9±0.7^{a,Z}
Analysis within the type of production								
Raw milk *fènè* production at home-scale	4.8±0.6	30.4±6.4	31.7±3.3^{a}	9.0±0.6^{a,Y}	9.0±0.5^{a,Y}	8.7±0.4^{a,Y}	9.1±0.5^{a,Y}	7.9±0.4^{a,b,Z}
Heated milk *fènè* production at home-scale	4.5±0.4	30.4±6.4	34.8±5.9^{b}	8.8±0.8^{a,Z}	9.0±0.9^{a,Z}	8.3±1.0^{a,Z}	9.2±0.3^{a,Z}	8.0±0.7^{a,Z}
Heated milk *fènè* production at small-scale	4.5±0.2	30.6±4.2	33.7±5.4^{a,b}	8.3±0.7^{a,Z,Y}	8.5±0.7^{a,Y}	8.2±0.8^{a,Z,Y}	8.5±0.7^{a,Y}	7.1±0.4^{b,Z}

$^{a, b}$: Column values with different letters (season or type of production) are significantly different by Tukey's test (p = 0.05). Data are means and standard deviations of six repetitions.

^{X,Y,Z}: Row values with different letters (agar media) are significantly different by Tukey's test (p = 0.05). Data are means and standard deviations of six repetitions.

* Temperatures recorded at each sampling point in the correspondent season or within the type of production.

P. pentosaceus was detected during the small-scale heated milk fermentation process in the samples Lm, F2 and F4 with 50%, 31% and 14%, respectively (results not displayed). *W. confusa* was found in small numbers during the same fermentation process in raw milk, F2 and F4 with 6% to 13% and together with *Lb. fermentum* both with 5% in the final product (Ff) (results not displayed). *Lc. lactis* subsp. *lactis* were only detected in raw milk (Lm), after 18 h (F18) and in the final product (Ff) with 13%, 9% and 5%, respectively in small-scale dairy *fènè* fermentation (Figure 4, C2).

2.5.3 Molecular typing at genus, species and strain level

A total of 1583 catalase-negative, Gram-positive cocci and rods were isolated during three seasons at domestic and small-scale *fènè* productions and partly typed down to species or subspecies and strain level (Table 6) with PCR, RFLP and rep-PCR (Figure 3). Rep-PCR displayed identical strain clustering than PFGE for the tested 37 isolates. We therefore used rep-PCR for strain clustering of isolates already typed to species level.

Of these 1583 isolates, 596 isolates were typed as enterococci, 454 as streptococci, 77 as *Lactobacillus fermentum* (typed into 18 different strains), 75 as *Weissella confusa* (21 strains), 62 as *Lactococcus lactis* subsp. *lactis* (21 strains), 36 as *Pediococcus pentosaceus* (30 strains), 24 as *Lb. plantarum* (9 strains), 11 as *W. paramesenteroides* (3 strains), 11 as *Lc. garvieae*, 10 as *Lactobacillus* sp., 8 as *P. acidilactici* (4 strains), 2 as *Lb. delbrueckii* subsp. (2 strains) and 217 isolates could not be identified.

213 of these 1583 isolates were isolated from the fermentation vat or surrounding air at the dairy during cold and rainy seasons (Table 6). Enterococci and streptococci were found most frequently in the environmental microbiota during cold and rainy season with 81 and 39 isolates respectively. *Lb. fermentum* (22 isolates), *Lb. plantarum* (19), *Lactobacillus* sp. (3), *W. paramesenteroides* (1), *W. confusa* (6) and *P. pentosaceus* (7) during cold season and *P. acidilactici* (2) during rainy season were identified in environmental samples. Thirty three isolates from both seasons remained unidentified at this point.

All 454 streptococci were previously analyzed with *St. thermophilus* specific PCR (Lick et al. 1996) (Figure 3). Seventy nine of 454 streptococci isolates testing positive in the *St. thermophilus* specific PCR (Lick et al. 1996) were grouped into 10 strain clusters with rep-PCR (Figure 5, Table 6). These ten putative *St. thermophilus* strain clusters

Table 5 Bacterial counts* [Log_{10}(cfu/ml)] during three different types of spontaneous *fènè* fermentation processes analyzed during hot, cold and rainy seasons for two repetition in one week interval

	Raw milk (sample Lm)			Start of the fermentation (F0)			2 hours of the fermentation (F2)		
	Hot season	Cold season	Rainy season	Hot season	Cold season	Rainy season	Hot season	Cold season	Rainy season
Raw milk production at home-scale	$5.5\pm0.5^{a,Z}$	$3.7\pm0.5^{a,Y}$	$4.2\pm0.5^{a,Y}$	$6.5\pm0.3^{a,Z}$	$4.4\pm0.8^{a,Y}$	$4.7\pm0.7^{a,Y}$	$7.1\pm0.5^{a,Z}$	$5.3\pm0.6^{a,Y}$	$4.6\pm0.8^{a,Y}$
Heated milk production at home-scale	$5.5\pm0.5^{a,Z}$	$3.7\pm0.5^{a,Y}$	$4.2\pm0.5^{a,Y}$	$2.4\pm0.8^{b,Z}$	$2.2\pm0.6^{b,Z}$	$2.8\pm0.1^{b,Z}$	$2.8\pm0.5^{b,Z}$	$2.5\pm1.0^{b,Z}$	$3.4\pm0.9^{b,Z}$
Heated milk production at small-scale	$6.9\pm0.6^{b,Z}$	$6.3\pm0.3^{b,Z}$	$6.0\pm0.3^{b,Z}$	$2.6\pm1.1^{b,Z}$	$2.1\pm0.5^{b,Z}$	$3.0\pm0.9^{b,Z}$	$2.8\pm0.9^{b,Z}$	$3.2\pm0.7^{b,Z}$	$3.4\pm1.0^{a,b,Z}$
	4 hours of the fermentation (F4)			End product *fènè* (Ff) (after 22 h)					
	Hot season	Cold season	Rainy season	Hot season	Cold season	Rainy season			
Raw milk production at home-scale	$8.0\pm0.4^{a,Z}$	$7.0\pm0.5^{a,Y}$	$6.1\pm1.0^{a,X}$	$9.3\pm0.4^{a,Z}$	$9.0\pm0.3^{a,Z,Y}$	$8.1\pm1.1^{a,b,Y}$			
Heated milk production at home-scale	$5.0\pm0.7^{b,Z}$	$4.3\pm1.0^{b,Z}$	$4.6\pm0.9^{a,Z}$	$9.2\pm0.4^{a,Z}$	$9.2\pm0.4^{a,Z}$	$8.5\pm0.3^{a,Y}$			
Heated milk production at small-scale	$4.4\pm1.0^{b,Z}$	$5.1\pm0.8^{b,Z}$	$4.7\pm1.6^{a,Z}$	$9.1\pm0.4^{a,Z}$	$8.4\pm0.2^{b,Y}$	$7.6\pm0.3^{b,X}$			

* Data are means and standard deviations of bacterial counts on non-selective agar media of eight repetitions.

$^{a, b}$: Column values with different letters (type of production) are significantly different by Tukey's test ($p = 0.05$).

^{X,Y,Z}: Row values with different letters (season) are significantly different by Tukey's test ($p = 0.05$).

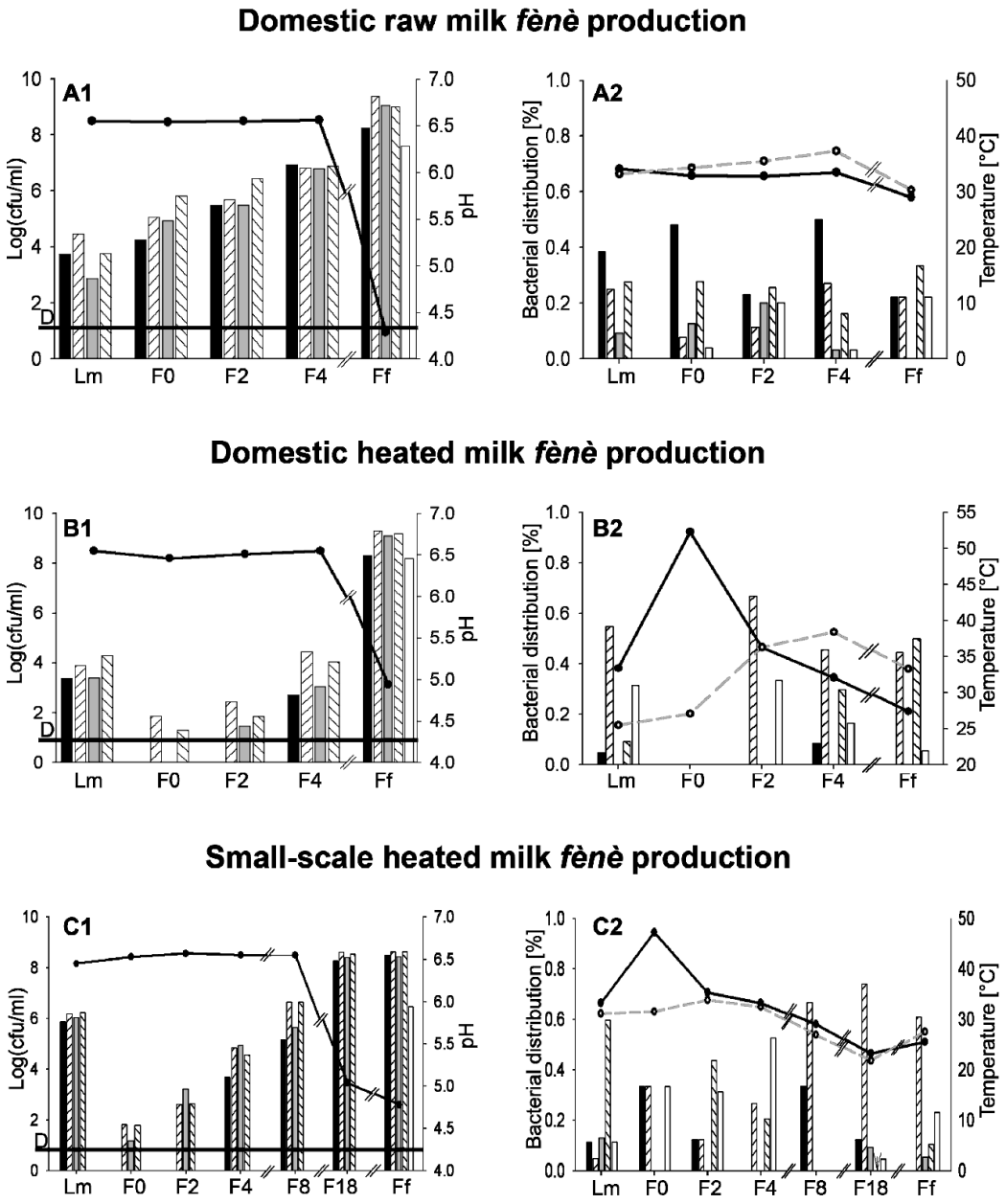

Figure 4 The fate of microbiota during spontaneous *fènè* fermentation processes.
At domestic raw milk *fènè* production (A), domestic heated milk *fènè* production (B) and small-scale heated milk *fènè* production (C) during the cold season with bacterial counts on KF (■), M17 (▨), MRS (▒), PC (▧) and VRBG (□) (only used for the final product (Ff)) agar plates and pH (—●—) in the graphics A1, B1 and C1 and relative bacterial distribution [%] obtained after molecular typing of genera and species isolated from not acidified MRS-agar plates (*Enterococcus* sp. ■; *Streptococcus* sp. and *St. bovis* ▨; *Lc. lactis* subsp. *lactis* ▒; *Lb. fermentum, W. confusa* and *P. pentosaceus* ▧; not typed isolates and small numbers of *Lc. garivea, W. paramesenteroides, P. acidilactici, Lb. plantarum* and *Lactobacillus* sp. □) and temperatures (—○— ambient; —●— milk) in the graphics A2, B2 and C2.
D = detection limit; Abbreviations of the sampling points: Lm: pooled milk; F0: start point of the spontaneous fermentation; F2, F4, F8, F18: 2h, 4h, 8h, 18h after fermentation started; Ff: final product (approximately 22 h after fermentation started).

different rep-PCR patterns than *St. thermophilus* DSM 20617^T and *St. thermophilus* DSM 20259 (Figure 5). Between 500 bp and 1450 bp of the 16S rRNA gene sequence of the isolates 11FA-1, 6C, 6BY-11b, 3AG, 11EQ-2, 11GJ-2; 8DF-1; 13AF; 13DW and 13AY-1, each representing one strain cluster of *St. thermophilus*, were sequenced, displaying homologies of over 99.0% with *St. infantarius* subsp. *infantarius* in gene bank data base matching while exhibiting maximum homology of 94% (isolate 13CH) to *St. thermophilus* DSM 20617^T in direct sequence comparison. Eight isolates selected randomly from 375 streptococci that tested negative in *St. thermophilus* specific PCR (Lick et al. 1996) were subjected to 16S rRNA gene sequencing. These eight streptococci isolates exhibited over 98% 16S rRNA gene sequence homology to (*St. infantarius* subsp. *infantarius.* Out of a total of 454 streptococci 87 were consequently identified as *St. infantarius* subsp. *infantarius*. One may therefore assume that *St. infantarius* subsp. *infantarius* is the dominant species of streptococci isolated from spontaneous *fènè* fermentation processes.

2.5.4 Strain diversity of spontaneous *fènè* fermentation processes

A total of 126 different strains from 384 typed isolates belonging to 10 different species were found in *fènè* samples collected during hot, cold and rainy seasons (Table 6). The highest strain diversity levels were observed for *P. pentosaceus* with 30 strains out of 36 isolates, whereas *St. infantarius* subsp. *infantarius* exhibited lowest strain diversity with 10 strains out of 79 isolates. Environmental microbiota (from fermentation vats/surrounding air) were tested in parallel at small-scale *fènè* fermentation processes. One *P. acidilactici* strain and four *Lb. fermentum* strains were found both in the environmental microbiota and the microbiota of the corresponding small-scale *fènè* fermentation process.

Strain detection within a single *fènè* fermentation process varied with respect to time as follows: only seven (one *Lc. lactis* subsp. *lactis,* one *St. infantarius* subsp. *infantarius,* three *W. confusa* and two *Lb. fermentum*) *strains* were detected both in the first eight hours of a spontaneous fermentation process and in the corresponding final product (*fènè*) from 18 spontaneous fermentation processes sampled in three seasons. Six strains (one *Lc. lactis* subsp. *lactis,* one *St. infantarius* subsp. *infantarius,* two *W. confusa* and two *W. paramesenteroides*) were detected only in the beginning (first eight hours) of a spontaneous fermentation process, whereas 27 strains (seven *Lc. lactis* subsp. *lactis,*

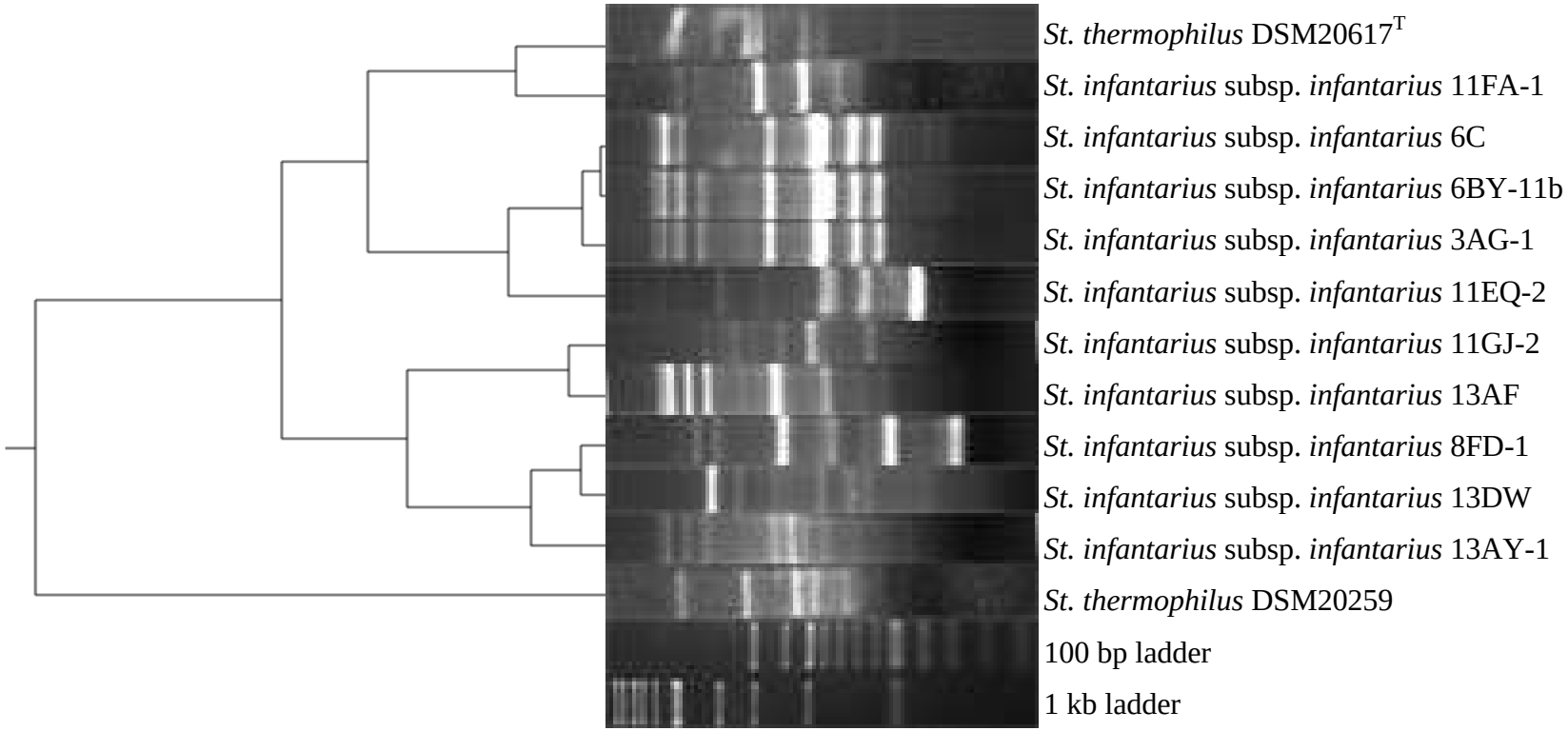

Figure 5 Statistical mapping of rep-PCR binding patterns of *St. infantarius* subsp. *infantarius* isolated from spontaneous *fènè* fermentation processes against *St. thermophilus* via the unweighted pair-group method using arithmetic averages clustering algorithm (UPGMA) (Garcia-Vallve et al., 2000; Sneath and Sokal, 1973; Tamura et al., 2007).

three *St. infantarius* subsp. *infantarius,* eight *P. pentosaceus,* seven *W. confusa* and two *Lb. fermentum*) were only found in the final products (*fènè*). 86 strains among 126 strains were only isolated once during spontaneous fermentation processes or from environmental samples.

Table 6 Lactic acid bacteria typed to strain level isolated from three types of spontaneous *fènè* fermentation processes at domestic- and small-scale during hot, cold and rainy seasons and environmental isolates (in parenthesis) form fermentation vats and surrounding air at small-scale production during cold and rainy seasons

Bacterial species	Total isolates	Hot season	Cold season	Rainy season	Number of strains per species
Lactobacillus sp.[a]	10	0	8 (3)	2	8
Lb. fermentum	77	10	64 (22)	3	18
Lb. plantarum	24	0	21 (19)	3	9
Lb. delbrueckii subsp.	2	2	0	0	2
Lc. lactis subsp. *lactis*	62	3	55	4	21
Lc. garvieae	11	0	11	0	–
Streptococcus sp. [b]	454	33	377 (38)	44 (1)	10 [c]
W. paramesenteroides	11	0	6 (1)	5	3
W. confusa	75	4	68 (6)	3	21
P. acidilactici	8	0	3	5 (2)	4
P. pentosaceus	36	0	35 (7)	1	30
Enterococcus sp.	596	124	356 (63)	116 (18)	–
not typed	217	7	153 (28)	57 (5)	–
Total isolates (strains)	1583	183	1157	243	(126)

Abbreviations of genera are indicated in Table 2.

– = value not determined.

[a] Lactobacilli which could not be typed to species level (results not shown).

[b] In total 87 streptococci isolates were typed as *St. infantarius* subsp. *infantarius* with PCR (Lick et al. 1996), rep-PCR and 16S rRNA gene sequencing.

[c] 79 streptococci isolates were typed with PCR (Lick et al. 1996) and 16S rRNA gene sequencing as *St. infantarius* subsp. *infantarius* and clustered with rep-PCR into ten strain clusters.

Strain diversity fluctuated greatly with only three strains (two *Lc. lactis* subsp. *lactis,* one *St. infantarius* subsp. *infantarius*) found in both the first and the second fermentation process sampled during a single season and seasonally with only four out of 126 strains

isolated at least in two seasons. These four strains included one *Lb. plantarum* and three *St. infantarius* subsp. *infantarius* strains present in two seasons, with one strain of *St. infantarius* subsp. *infantarius* found in all three seasons. Seven strains (two *Lc. lactis* subsp. *lactis,* one *St. infantarius* subsp. *infantarius,* two *W. confusa* and two *Lb. fermentum*) were found at both domestic and small-scale production sites during a single season (results not displayed).

2.6 Discussion

Isolation and typing of large numbers of bacteria of unknown origins is still laborious and challenging work. A process facilitating typing lactic acid bacteria (LAB) to strain level was applied by combining morphological and phenotypic tests and known molecular biological methods into a binary flow chart (Figure 3). Seasonal and production environment-dependent variation of microbiota diversity, critical entry points of fermentative or contaminating microbes and growth dynamics of microbial populations were investigated with the binary model typing process introduced in this work.

The PCR and RFLP protocol to identifying enterococci (Ke et al. 1999), lactobacilli (Dubernet et al. 2002; Song et al. 2000), pediococci (Mora et al. 1997) and *Weissella* sp. (Jang et al. 2002) was incorporated directly into our typing approach. Some lactococci identified by PCR at genus level (Deasy et al. 2000) could not be classified into unique *Lactococcus* species by multiplex PCR (Pu et al. 2002) (results not displayed). These putative lactococci not confirmed at species level often showed positive signals with the *Streptococcu*s genus specific PCR (28 species are included) developed by Picard et al. (2004) (results not displayed).

Bacterial counts of pooled raw milk at small-scale were at least 10-fold higher than found in raw milk at domestic production, due to milk transportation to the plant in plastic containers at ambient temperature and corresponded to other findings in the literature (Bonfoh et al. 2003c). *Enterobacteriaceae* counts remained at relatively consistent levels throughout the year, whereas levels of presumptive lactococci, lactobacilli and enterococci and aerobic mesophilic counts were, in comparison, significantly higher during hot and cold season. Microbial counts of all presumptive genera normalized around $1x10^8$ cfu/ml during rainy season. This relativization is not without consequence – a significant decrease of fermentative microbiota while *Enterobacteriaceae* counts remain constant (pH>4.5) raises the potential threat level of the latter to human health.

Spontaneous *fènè* fermentation processes vary seasonally and from one production environment to another. This is especially apparent at small-scale dairies when heated milk was inadvertently inoculated with opportunistic LAB through environmental contamination. Spontaneous fermentation initiated through rapid recontamination by environmental microbiota during milk cooling occurred after milk heating, with streptococci and enterococci being the first LAB colonizing cooled milk. These bacteria were persistently present in all successive samples analyzed whereas other LAB, were less regularly isolated from fermentation processes. Fluctuations in strain diversity were found from fermentation to fermentation during the same season, and may be traced to environmental contaminants or production hygiene. The fact that only a few strains were detected both at early stages of fermentation and in the final product underscores the importance of multiple sampling throughout complex fermentation processes.

The published PCR-assay for *St. thermophilus* (Lick et al. 1996) cannot differentiate *St. thermophilus* from *St. bovis* group. Ten isolates representing the ten strain clusters and the eight randomly selected isolates were subsequently typed as *St. infantarius* subsp. *infantarius* by PCR and RFLP (Chen et al. 2008). The *St. bovis* group specific PCR/RFLP (Chen et al. 2008) were therefore later integrated into our typing flow chart (Figure 3).

Microbiota composition of spontaneously fermented *fènè* depends on season and production environment and exhibits elevated biodiversity. Small-scale dairy fermentation was initiated by environmental contaminants and numerous lactobacilli, lactococci, *Weissella* sp. and pediococci, with a conspicuous absence of *St. thermophilus*. Enterococci and streptococci (*St. infantarius* subsp. *infantarius*) dominate *fènè* microbiota. *Lc. garvieae* another well known pathogen was detected in *fènè* microbiota as well. Bacterial counts of over 10^7 cfu/ml presumptive *Enterobacteriaceae* and over 10^5 cfu/ml presumptive *Staphylococcus aureus* further underscored these potential health hazards.

The typing protocol applied in this study has proven itself likewise invaluable determining the commensal microbial composition as well as pinpointing elevated levels of putative human pathogens in complex food matrices. We believe the elevated levels of putative human pathogens pose a potential health hazard to consumers of *fènè* and special attention should be paid to *St. bovis* (*St. infantarius* subsp. *infantarius*) since clinical isolates of bacteria belonging to the *St. bovis* / *St. equinus* complex are known to cause bacteremia, endocarditis and may trigger colon and non-colon cancers (Corredoira et al.

2008a; Corredoira et al. 2008b; zur Hausen 2006). The introduction of an adapted starter culture in concert with improved production hygiene while monitoring critical control points can assist in the safe production of *fènè* locally. To these ends, profiles of *fènè* microbiota have been conserved to aid in the development of these starters.

2.7 Acknowledgement

We acknowledge the contribution of Zakaria Farah (Laboratory of Food Biotechnology, ETH Zurich, Switzerland), Jakob Zinsstag (Department of Epidemiology and Public Health, Swiss Tropical Institute, Basel, Switzerland) and Saïdou Tembely (Laboratoire Central Vétérinaire (LCV), Bamako, Mali). This project was financially supported by ETH Zurich internal funds, the Commission for Research Partnership with Developing Countries (KFPE, Bern, Switzerland) and the Walter Hochstrasser Foundation (Zurich, Switzerland). We also acknowledge the Swiss National Centre of Competence in Research (NCCR) North–South for providing the research platform support through the Institute of Sahel.

3 Seasonal-dependent antibiotic resistance prevalence of enterococci from the production of *fènè*, a spontaneously fermented milk of Mali

Data presented in this chapter are submitted for publication to an international peer-reviewed journal in 2009 with the authorship:
Stephan Wullschleger, Alexandra Dostal, Salome Egger, Bassirou Bonfoh, Christophe Lacroix, Vincent Perreten and Leo Meile

3.1 Abstract

Enterococci (n=118) were isolated from small-scale production of spontaneously fermented sour-milk *(fènè)* during hot, cold and rainy seasons in a village near Bamako (Mali). They were analyzed for phenotypic and genotypic antibiotic resistance (AR) and for transferability of acquired resistance genes. All isolates were susceptible against ampicillin, chloramphenicol and gentamicin and only a few strains (between 3% and 7% per season) displayed decreased susceptibility against erythromycin and vancomycin with inhibition halos close below the corresponding resistance borderlines but any findings of related acquired resistance genes. The AR prevalence against tetracycline and oxytetracycline (between 13% and 40%) was related to the amount of oxytetracycline purchased during the preceding season. Both tetracycline resistance genes *tet*(L) and *tet*(M) were detected in 85% of tetracycline resistant enterococci. The sole occurrence of either the *tet*(M) or *tet*(S) genes was found in the remaining tetracycline resistant enterococci. Only one from 20 tetracycline resistant enterococci isolates transferred simultaneously the *tet*(L) and *tet*(M) genes into *E. faecalis* JH-2-2 ($1.9*10^{-6}$ transconjugants per donor) by conjugation.

3.2 Introduction

The use of antimicrobial agents in animal husbandry is small in West Africa compared to industrialized countries. When used, farmers often neglect withdraw times after antibiotic treatment due to small income, missing knowledge and inadequate legislation. Uncontrolled drug use including sub-inhibitory antibiotic concentrations (Levy and Marshall 2004) is probably the major factor contributing to the selection of AR in food related bacteria in developing countries. Oxytetracycline and streptomycin are the most frequently used antibiotics in Mali (Bonfoh et al. 2003a) and are available at pharmacies without prescription. Residues of antibiotics were detected in two series of 202 and 72 milk samples in urban Bamako the capital of Mali at 6% and 16%, respectively in a previous study (Bonfoh et al. 2003a). Antibiotic residues in milk were less frequently detected during the rainy season (June to September) than during hot and cold seasons (Bonfoh et al. 2003a). These findings indicate an inappropriate use of antibiotics in animal husbandry in Mali and suggest the generation and spreading of antibiotic resistant bacteria in the food chain (Aarestrup 2005) and a subsequent transmission to human beings is likely (Okeke et al. 2005).

The AR situation is well documented for enterococci from food origin in developed countries (Aarestrup 2005; Giraffa 2002; Gomes et al. 2008; Huys et al. 2004; Leisibach 2004; Macovei and Zurek 2007; Wilcks et al. 2005). However, only a few AR studies have been conducted on bacteria originating from traditional African ready-to-eat food products (Chingwaru et al. 2003; Getahun et al. 2008). We therefore isolated enterococci from spontaneous small-scale milk fermentations leading to *fènè*, a traditional sour milk product of Mali and analyzed their phenotypic and genotypic AR profiles during the hot, cold and rainy seasons.

3.3 Material and methods

3.3.1 Bacterial strains

Enterococci were isolated on Azide maltose agar (KF) (Labo-Life Sàrl, Pully, Switzerland) during the cold (November and December 2005), hot (February and March 2005) and rainy (June and July 2006) seasons each time from two independent *fènè* fermentation processes and raw milk separated by a one week interval (Chapter 2) and typed to genus level by PCR (Ke et al. 1999). *Fènè* is a popular artisanal fermented milk of Mali and was produced for this study from heat-treated cow milk following spontaneous overnight fermentation at the small-scale dairy Bagan Yiri Wa Ton in Kasséla, a village 40 km from Bamako the capital of Mali as described elsewhere (Chapter 2). Analysis of phenotypic and genotypic antibiotic resistance properties was performed with 58 out of 68, 30 out of 96 and 30 out of 66 enterococci from the hot, cold and rainy seasons, respectively. *Enterococcus faecalis* DSM 2570 (=ATCC 29212) (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) harboring the *tet*(M) gene (this study), *E. faecalis* SL89.10 [*tet*(M) and *tet*(L)] (Leisibach 2004) and *E. faecalis* JH2-2 (Jacob and Hobbs 1974) were used as control strains for phenotypic and genotypic antibiotic profiling as well as for filter mating experiments.

3.3.2 Phenotypic antibiotic resistance profiling

Enterococci were grown in Brain Heart Infusion (Labo-Life Sàrl) overnight at 37℃, then standardized to an optical density of 0.125 (λ = 550 nm, Uvikon 810 P, Tegimenta Ag, Rotkreuz, Switzerland) and streaked to a dense layer with sterile cotton taps for disc diffusion tests on Mueller Hinton agar plates (Labo-Life Sàrl). The following antibiotic discs (BioMérieux, Geneva, Switzerland, and Oxoid, Basel, Switzerland) were used: ampicillin (10 µg), bacitracin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg),

fosfomycin (50 µg), gentamycin (10 µg), norfloxacin (10 µg), novobiocin (5 µg), oxytetracycline (30 µg), penicillin G (10 units), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). Assessment of resistance breakpoints was done according to the CLSI guidelines for enterococci (CLSI 2006b). No guidelines were available for bacitracin, novobiocin and oxytetracycline and therefore the disc diameter itself was chosen as reference diameter for resistance. Strain *E. faecalis* DSM 2570 (equivalten to ATCC 29212) was used as control for breakpoint data (CLSI 2006b).

3.3.3 Genotypic antibiotic resistance profiling

AR genes in *Enterococcus* isolates were identified by a microarray hybridization assay targeting ninety different AR genes (Perreten et al. 2005) and using randomly labeled DNA. Detected AR genes were confirmed by PCR (Table 7).

3.3.4 Transferability of antibiotic resistance genes

Genetic transfer experiments were done by filter mating with *E. faecalis* JH2-2 (Jacob and Hobbs 1974) as recipient strains. *E. faecalis* SL 89.10 (Leisibach 2004) was used as a positive control donor strain for tetracycline resistance transfer. Transconjugants (TCs) were streaked out twice on plates containing 10 µg/ml tetracycline, 50 µg/ml rifampicin and 100 µg/ml fusidic acid and transferred resistance genes were confirmed by PCR.

Table 7 Oligonucleotide primers and PCR conditions used to confirm the tetracycline resistance genes *tet*(M), *tet*(L), *tet*(S) and the resistance gene *msr*(C) and *aac(6')-Ii*

Target gene	Primer	Sequence of primers 5' → 3'	PCR conditions (35 cycles)*	Amplicon length	Positive control	Reference
tet(M)	Tet1 Tet2	GCTCATGTTGATGCAGGA AGGATTTNNCGGCACTTCGAC	95℃ 30 sec, 54℃ 30 sec, 72℃ 1 min;	1293 bp	*E. faecalis* 89.10	(Leisibach 2004)
tet(L)	TetL1F TetL1R	CCTGCGAGTACAAACTGG TCAAGGTAACCAGCCAAC	95℃ 30 sec, 54℃ 30 sec, 72℃ 1 min;	1203 bp	*E. faecalis* 89.10	(Leisibach 2004)
tet(S)	TetS-1 TetS-2	ATCAAGATATTAAGGAC TTCTCTATGTGGTAATC	95℃ 30 sec, 54℃ 30 sec, 72℃ 1 min;	573 bp	plasmid pK214 (Perreten et al. 1997)	(Leisibach 2004)
msr(C)	msrC-F msrC-R	TTGGAACATATCCGCAAACA CGGATATACTTGCATCAGC	95℃ 30 sec, 57℃ 30 sec, 72℃ 1 min;	351 bp	none	this study
aac(6')-Ii	aac(6)-Ii-F aac(6)-Ii-R	GAGATACTGATTGGTAGC GAGATACTGATTGGTAGC	95℃ 30 sec, 50℃ 30 sec, 72℃ 1 min;	1300 bp	*E. faecium* 70/90 (Klare et al. 1995)	(Perreten et al. 2005)

* Initial denaturation at 95℃ for 5 minutes and final polymerization at 72℃ for 7 minutes were applied for all PCR.

3.4 Results

3.4.1 Phenotypic antibiotic resistance profiling and seasonal differences

Enterococci were isolated from different sampling points of six independent spontaneous small-scale *fènè* fermentation processes and raw milk and reached in the final product on KF agar 9.0±0.5, 8.3±0.2 and 7.3±0.1 Log_{10}(cfu/ml) in the hot, cold and rainy seasons, respectively. Phenotypic AR profiles of 118 enterococci isolated from different seasons were analyzed by disc diffusion tests (Table 8). All (n=118) enterococci were susceptible against ampicillin, chloramphenicol, gentamicin and norfloxacin and only a few enterococci with a maximum of 7% per season displayed lower susceptibility against bacitracin, ciprofloxacin, doxycyline, erythromycin, novobiocin and vancomycin (Table 8).

Table 8 Phenotypic antibiotic resistance prevalence of 118 enterococci isolated from tow spontaneous small-scale *fènè* fermentations per season during the cold, hot and rainy seasons in Mali.

No antibiotic resistance prevalence was found against ampicilin, chloramphenicol, gentamycin and norfloxacin

Antibiotic	***E. faecalis* DSM 2570**	**Seasonal prevalence of AR enterococci**		
		cold season (n=30)	**hot season (n=58)**	**rainy season (n=30)**
Bacitracin	S	0%	n. t.	3%
Ciprofloxacine	S	0%	n. t.	3%
Doxycycline	S	7%	n. t.	0%
Erythromycin	S	7%	0%	0%
Fosfomycin	S	3%	n. t.	17%
Novobiocin	S	3%	n. t.	0%
Oxytetracycline	S	37%	23%	13%
Penicillin	S	3%	16%	0%
Streptomycin	R	93%	70%	100%
Tetracycline	R	40%	26%	17%
Vancomycin	S	7%	0%	0%

n.t. = not tested.

High penicillin resistance prevalence was detected during hot season with 16% and high fosfomycin prevalence resistance during rainy season with 17%. Highest resistance prevalence against oxytetracycline and tetracycline were detected in strains isolated during cold season with 37% and 40%, less with 23% and 26% during hot season and fewest during rainy season with 13% and 17%, respectively.

3.4.2 Genotypic antibiotic resistance profiling

Twenty one isolates with phenotypic erythromycin, tetracycline or vancomycin resistances were investigated on acquired resistance genes (Table 9). In 18 out of 20 phenotypic tetracycline resistant enterococci the ribosomal protection gene *tet*(M) was detected. In 17 out of the 18 *tet*(M) harboring enterococci the *tet*(M) gene was detected in combination with the *tet*(L) gene encoding energy-dependent efflux pumps. *Tet*(M) alone was detected in strain 26DE isolated from raw milk during the rainy season. Strain 6BR and 13DT-22 both isolated from raw milk prior to the heating step during the hot and cold season, respectively, harbored at least the *tet*(S) gene (Figure 6). The *msr*(C) gene, which is reported to be indigenous for *E. faecium* and is reported to encode an erythromycin efflux pump (Portillo et al. 2000; Singh et al. 2001), was detected in 19 of 21 analyzed strains. No acquired erythromycin or vancomycin resistance genes were detected in strains exhibiting phenotypic resistances to these antibiotics. The gene *aac*(6')_*li,* encoding for an acetyltransferase, was detected in all nine tetracycline resistant strains from the hot season and in five of the nine tetracycline resistant strains from the cold season but could not be confirmed by PCR.

3.4.3 Transferability of antibiotic resistance genes

The 20 enterococci strains harboring tetracycline resistance genes were submitted to filter mating experiments with the recipient strain *E. faecalis* JH-2-2 (Jacob and Hobbs 1974). Strain *E. faecalis* SL.89.10 (Leisibach 2004) was used as a positive donor. This strain harboring the *tet*(M) and *tet*(L) genes transferred in our experiments only its *tet*(L) gene with a transfer rate of $2.3*10^{-4}$ TCs per donor to *E. faecalis* JH-2-2. Strain 27Q, isolated after 4 hours of the spontaneous *fènè* fermentation process during the rainy season, was the only one which transferred both its *tet*(M) and *tet*(L) genes with a transfer rate of $1.9*10^{-6}$ TCs per donor to *E. faecalis* JH2-2.

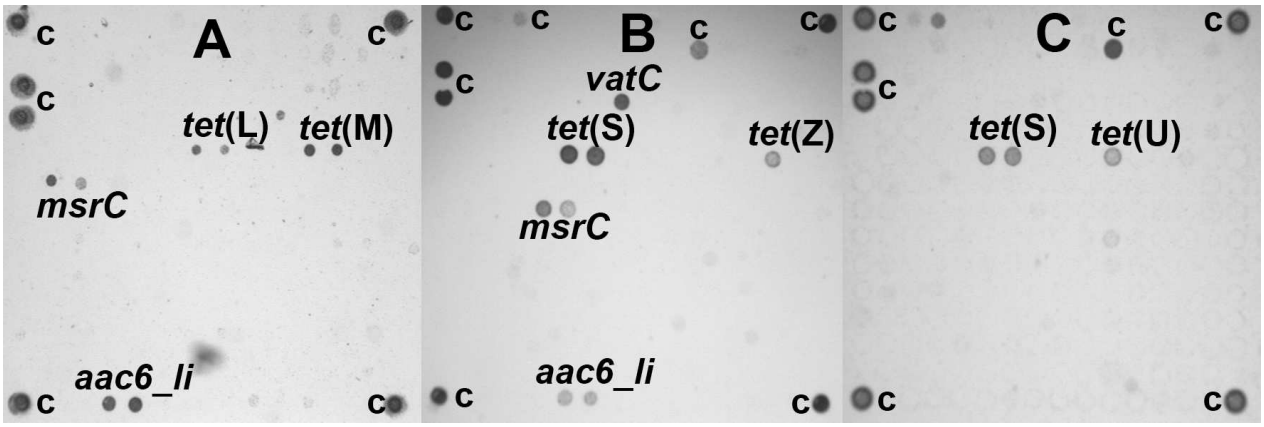

Figure 6 Microphotographs of microchip hybridization assays with DNA from the enterococci strains 6BO (A), 6BR (B) and 13DT-22 (C).

c biotin position reference markers.

Table 9 Genotypic and phenotypic antibiotic resistance profiles of selected enterococci isolated form spontaneous small-scale *fènè* fermentations during the cold, hot and rainy seasons in Mali

Strain	Season[a]	Source[b]	cfu/ml[c]	Phenotypic antibiotic resistance profile[d]	Resistance genes detected by micro-array hybridization[e]	Resistance gene confirmed by PCR[f]
5A-11	h	Lm	$7.15*10^5$	T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
5A-3	h	Lm	$7.15*10^5$	T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
5B-3	h	Lm	$7.15*10^5$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
5BX-141	h	F4	$4.05*10^5$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
5BX-1421	h	F4	$4.05*10^5$	T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
5BX-1422	h	F4	$4.05*10^5$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
6BR	h	Lm	$1.39*10^6$	T^r, TE^r	*tet*(S), *msrC*, *aac(6')-Ii*	*tet*(S), *msr*(C)
6BO	h	F2	$4.00*10^1$	T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
6BV-1	h	F2	$4.00*10^1$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
8FK	c	Ff	$2.29*10^8$	E^r, P^r, S^r, T^r, TE^r, VA^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
8FN	c	Ff	$2.29*10^8$	E^r, S^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
8FS-2	c	Ff	$1.73*10^8$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C)	*tet*(L), *tet*(M), *msr*(C)
8FE	c	Ff	$1.73*10^8$	FF^r, S^r, T^r, VA^r	*msrC*, *aac(6')-Ii*	*msr(C)*
8FI	c	Ff	$1.73*10^8$	D^r, S^r, TE^r	*tet*(L), *tet*(M), *msr*(C)	*tet*(L), *tet*(M), *msr*(C)
8EY-1	c	Ff	$1.73*10^8$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C)	*tet*(L), *tet*(M), *msr*(C)
8EK	c	Ff	$1.73*10^8$	D^r, S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
13DT-22	c	Lm	$7.38*10^5$	T^r, TE^r	*tet*(S)	*tet*(S)
13EH-2	c	F2	$1.63*10^3$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C) , *aac(6')-Ii*	*tet*(L), *tet*(M), *msr*(C)
26DE	r	Lm	$1.03*10^5$	CIP^r, S^r, TE^r	*tet*(M)	*tet*(M)
27FI	r	F18	$6.20*10^6$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C)	*tet*(L), *tet*(M), *msr*(C)
27Q	r	F4	$1.94*10^5$	S^r, T^r, TE^r	*tet*(L), *tet*(M), *msr*(C)	*tet*(L), *tet*(M), *msr*(C)

[a] h: hot season; c: cold season; r: rainy season.

[b] Sources are spontaneous *fènè* fermentation processes. Abbreviations indicate the sampling point within one process: Lm: raw milk before a heating step of up to 85°C and re-cooling at ambient temperature; F2: 2 h after fermentation started (fermentation started when milk was colder than 50°C); F4: 4 h after fermentation started; F18: 18 h after fermentation started; Ff: final product (approximately 22 h after fermentation started).

[c] cfu/ml on KF agar of corresponding samples.

[d, r] Indicates resistance to: CIP ciprofloxacine, D doxycycline, E erythromycin, FF fosfomycin, P penicillin, S streptomycin, T oxytetracycline, TE tetracycline, VA vancomycin.

[e] Genes encode for the following resistances: *tet*(L), *tet*(M), *tet*(S) tetracycline; *msr*(C) erythromycin or are indigenous for *E. faecium*; *aac(6')-Ii* some aminoglycosides (Perreten et al. 2005; Portillo et al. 2000; Singh et al. 2001).

[f] According to PCR protocols displayed in Table 7.

3.5 Discussion

Particular food from the agro-food chain including antibiotic treatment steps of animals and plants and lacking final heating steps (Perreten et al. 1997) are predestined to harbor high numbers of AR bacteria. Our sampling material, *fènè*, belongs to this risk group since the initial milk is exposed after a heating step to high environmental microbial contamination, which also serves as inoculation for the fermentation to *fènè* (chapter 2). Enterococci were isolated from artisanal fermented milks produced in Mali by a rural small-scale dairy during the three seasons of a year. Enterococci has been used as bacteria to investigate AR occurrence in food and food-producing animals, because enterococci act as turntable for AR genes (Teuber et al. 1999). Antibiotic surveillance should be conducted on a long time space in order to draw some conclusions about the development of AR occurrence in a particular area (e.g. agriculture; food; milk). The antibiotic use in animal husbandry in Mali varies between the cold, hot and rainy season due to pathology reason of milk cows. Most antibiotics are sold during the rainy season, less during the cold season and fewest during the hot season. Seasonal influence on tetracycline and oxytetracycline resistance events occurs delayed. Most tetracycline resistant enterococci were consequently not found in the rainy season but in the following cold season. Less AR enterococci were found in the hot season, which follows the cold season. In the rainy season, which follows the hot season and is also the season most antibiotics are sold, the fewest AR enterococci were detected. This is in correlation with the findings that in the rainy season fewest antibiotic residues in local milk were found (Bonfoh et al. 2003a) and is explicable by the fact, that farmer purchase antibiotic mostly during the rainy season but subsequent treatments are delayed to the cold season. Antibiotics are only used therapeutically in Mali and therefore, antibiotic pressure varies from season to season according to particular infections in animals. This fact and broad microbiota diversity allow a reversibility of AR occurrence within the following seasons probably due to a dilution effect (Levy and Marshall 2004) of not resistant bacteria substantiated through strong seasonal-dependent strain diversity in *fènè* microbiota, shown for lactic acid bacteria (chapter 2). Higher AR prevalence in enterococci are reported for industrialized countries (Chingwaru et al. 2003; de Fatima Silva Lopes et al. 2005; Giraffa et al. 2000; Leisibach 2004; Templer and Baumgartner 2007; Teuber 2001; Teuber et al. 1999) compared to this study, except for oxytetracycline and tetracycline, which have a similar mode of action,

and showed resistance levels comparable to industrialized countries (Huys et al. 2004), due to the frequent use of oxytetracycline in Mali (Bonfoh et al. 2003a).

The tetracycline resistant genes *tet*(M) and *tet*(L) are most often detected in enterococci from food origins (Hummel et al. 2007; Huys et al. 2004; Macovei and Zurek 2007; Wilcks et al. 2005). Combined *tet*(M) and *tet*(L) resistance was however in none of these studies reported at a similar high extent than in our study. A lower *tet*(S) frequency compared to the occurrence of the *tet*(M) and *tet*(L) genes was reported in this and other studies (Huys et al. 2004; Macovei and Zurek 2007). None of our analyzed enterococci harbored the *tet*(O) or *tet*(K) genes reported elsewhere (Hummel et al. 2007; Macovei and Zurek 2007). Whereas transfer experiments of *tet*(M) and *tet*(L) genes from food originating enterococci to *E. faecalis* recipient strains were successful in our and other recently published works (Hummel et al. 2007; Huys et al. 2004; Wilcks et al. 2005).

No corresponding acquired AR genes were found in the erythromycin or vancomycin resistant strains 8FN, 8FE, 8FK (Tables 2 and 3), explicable by their inhibition halos (12 mm or 13 mm) close the resistance borderline (CLSI 2006b). The high streptomycin resistance prevalence found in this study is probably more related to the fact that enterococci are low-level aminoglycoside resistant (Giraffa 2002) and not due to increased streptomycin use in Malian (Bonfoh et al. 2003c), because we detected no streptomycin resistance gene.

Our results suggest that AR is related to the antibiotic pressure applied, with tetracycline as an example in this study. We could confirm the general assumption that AR frequency in Mali is lower than in industrialized countries with phenotypic AR profiles of 15 antibiotics. The situation of antibiotic resistant bacteria in food of developing countries, originating from a agro-food chain with selective antibiotic pressure and lacking sufficient heat-treatment, may reach once the level of industrialized countries due to increasing property, allowing extended antibiotic treatments in local agriculture, if no corresponding education and legislation measures will be introduced.

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4 Antibiotic resistance of non-*Enterococcus* lactic acid bacteria from spontaneous sour milk *(fènè)* production in Mali

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Stephan Wullschleger, Sarah Baumgartner, Bassirou Bonfoh, Christophe Lacroix, Vincent Perreten and Leo Meile

4.1 Abstract

Antibiotic susceptibility profiles of 13 *Lactobacillus fermentum*, 9 *Lb. plantarum*, 9 *Lactococcus lactis* subsp. *lactis*, 4 *Pediococcus acidilactici*, 23 *P. pentosaceus*, 7 *Streptococcus infantarius* subsp. *infantarius*, 16 *Weissella confusa* and 3 *W. paramesenteroides* isolated from spontaneous small-scale and domestic sour milk (*fènè*) fermentation processes in rural Mali were analyzed. Selected strains were subsequently investigated for the presence of transferable antibiotic resistance genes by microarray hybridization with the further goal to develop safe adapted starter cultures for better controlled small-scale *fènè* fermentations. Resistance to tetracycline and oxytetracycline were found in 17% of the investigated strains. Four tetracycline resistant *Lc. lactis* subsp. *lactis* and the seven *St. infantarius* subsp. *infantarius* harbored the *tet*(S) gene, whereas a *Lb. plantarum* strain harbored the *tet*(M) gene. It suggests that sub-therapeutic oxytetracycline doses applied in local animal husbandry selected for a tetracycline resistance gene pool, which is transferable between different genera within the investigated environment and might spread to human beings.

4.2 Introduction

Antibiotic use in the agriculture of developing countries is small and applied only for therapeutic measures, but antibiotics are available without prescription and are mostly used inadequately in sub-therapeutic doses (Planta 2007). No governmental regulations on the use of antibiotic in agriculture are existent (Levy and Marshall 2004; Okeke et al. 2005). This uncontrolled antibiotic use finally contaminates the dairy food chain and antibiotic residues have been found in milk because farmer neglect withdraw times due too very low milk production and corresponding small revenues (Bonfoh et al. 2003a). The prevalence of tetracycline and oxytetracyline resistant enterococci from *fènè* microflora is related to the oxytetracyline pressure applied (chapter 3). Antibiotic resistant bacteria (ARB) are a well known worldwide risk on human health (Levy and Marshall 2004), which has recently been defined as a shadow epidemic (Ammor et al. 2007), and specially milk represents a source where resistant bacteria can enter the human food chain (Perreten et al. 1997).

Nowadays increased attention is paied to safety of bacteria applied in food. The EFSA proposed by the Qualified Presumption of Safety (QPS) approach only to use bacteria, which do not harbour any acquired antibiotic resistance genes unless they are due too chromosomal mutation

(Chamba and Jamet 2008; European Commission 2005). Breakpoints used for antibiotic susceptibility profiling are harmonized in Europe by the EUCAST but unfortunantely no harmonized guidelines for testing non-*Enterococcus* LAB are available and therefore results are not well comparable (European Commission 2005; Flórez et al. 2008).

In Africa, bacteria involved in milk fermentation have not yet been well characterized and their resistance profile is unknown. We investigated 84 non-*Enterococcus* LAB from the microbiota of *fènè,* a local sour milk of Mali produced by spontaneous fermentation of cow milk, on acquired antibiotic resistances by phenotypic and genotypic profiling with the objectives to generate additional knowledge on public safety hazards related to antibiotic resistant bacteria in traditional African foodstuff and to select suitable bacteria for subsequent development of safe adapted starter cultures for better controlled small-scale *fènè* fermentation.

4.3 Material and Methods

4.3.1 Bacterial strains and growth conditions

13 *Lactobacillus fermentum*, 9 *Lb. plantarum*, 9 *Lactococcus lactis* subsp*. lactis*, 4 *Pediococcus acidilactici*, 23 *P. pentosaceus*, 7 *Streptococcus infantarius* subsp. *infantarius*, 16 *Weissella confusa* and 3 *W. paramesenteroides* strains were isolated from spontaneous domestic and small-scale *fènè* fermentations and checked on their previous typing by PCR, RFLP and rep-PCR (chapter 2) for analysis within this study. The type strains *Lb. fermentum* DSM 20052^T (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), *Lb. plantarum* DSM 20174^T, *Lc. lactis* subsp. *lactis* DSM 20481^T, *P. acidilactici* DSM 20284^T, *P. pentosaceus* DSM 20336^T, *St. bovis* DSM 20480^T, *W. confusa* DSM 20196^T and *W. paramesenteroides* DSM 20288^T were incorporated into antibiotic susceptibility testing. *Staphylococcus aureus* DSM 2569, *Escherichia coli* DSM 1103 and *Enterococcus feacalis* DSM 2570 were used as controls (Klare et al. 2005). Bacteria were cultivated on MRS agar plates (Labo-Life Sàrl, Pully, Switzerland) anaerobic, except *E. faecalis* DSM 2570 and *S. aureus* DSM 2569 which were grown aerobic. *E. coli* DSM 1103 was grown aerobic on Brain Heart Infusion (Becton Dickinson, Allschwil, Switzerland) agar plates. *Lc. lactis* subsp. *lactis* and *W. paramesenteroides* strains were incubated at 30℃, the other bacteria at 37℃.

4.3.2 Phenotypic antibiotic resistance profiling

Disc diffusion tests and microdilution assays (Klare et al. 2005) were performed for phenotypic antibiotic profiling with the objective to relate both minimal inhibition concentration (MIC) data and disc diffusion diameters to each other. LSM media (Klare et al. 2005) was used in both tests, except for *St. infantarius* subsp. *infantarius* SSM was used (Tosi et al. 2007). Agar plates used in disc diffusion tests contained 1.5% Agar (Becton Dickinson). Bacteria were profiled with disc diffusion tests against: amoxicillin / clavulanic acid (antibiotic disc concentration [µg/disc]: 20/10), ampicillin (10), chloramphenicol (10), ciprofloxacin (5), clindamycin (2), erythromycin (15), fusidic acid (10), gentamicin (10), kanamycin (30), linezolid (30), mupirocin (200), neomycin (30), novobiocin (5), oxacillin (1), oxytetracycline (30), penicillin G (10 units), quinopristin/dalfopristin (15), rifampicin (30), streptomycin (10), tetracycline (30), trimethoprim / sulfamethoxazol (1.25/23.75) and vancomycin (30). Bacteria were therefore grown overnight, standardized to an optical density of 1.0 measured at 600 nm (Univok 810P photometer,

Flowspek AG, Basel, Switzerland) and streaked as a dense layer by sterile cotton swabs onto agar plates for disc diffusion tests. Inhibition zones were analyzed once and measured accurate to a millimetre and compared to CLSI guidelines for *Streptococcus* sp. other than *St. pneumoniae* (CLSI 2006a) for *St. infantarius* subsp. *infantarius* strains (Table 10). No guidelines were available for disc diffusion breakpoints of antibiotic-bacteria combinations not displayed in Table 10.

MIC of amoxicillin (test range [µg/ml]: 0.125-32), ampicillin (0.031-64), chloramphenicol (0.125-256), clindamycin (0.016-32), erythromycin (0.016-32), fusidic acid (0.016-128), gentamicin (0.25-64), kanamycin (0.5-128), mupirocin (0.063-128), nalidixic acid (0.125-256), novobiocin (0.031-64), penicillin G (0.031-64), rifampicin (0.125-64), streptomycin (0.5-256), tetracycline (1-256), trimethoprim (0.25-512) and vancomycin (0.125-256) were determined. Optical density (PowerWaveTM XS, BioTek®, Vermont, USA) of an overnight culture measuered at 600 nm was set to 0.4 for micro-dilution tests. The tests were performed in 150 µl onsets in 96 well microtiter plates inoculated with 10^5 cfu/ml (Klare et al. 2005). The experimental approche was validated by twofold repetition with *E. coli* DSM 1103, *S. aureus* DSM 2569 and *Enterococccus faecalis* DSM 2570 on ten antibiotics. Minimal inhibitory concentration (MIC) values were in agreement to data published by Klare et al. (2005). The experimental approach was validated by twofold repetition with *E. coli* DSM 1103, *S. aureus* DSM 2569 and *E. faecalis* DSM 2570 on ten antibiotics. MIC values were in agreement to data published by Klare et al. (2005). The MIC was defined as half of the optical density of a particular type strain grown in antibiotic free media at growth conditions described herein. Strains were declared as resistant after comparing their MIC values to guidelines (CLSI 2006a; European Commission 2005; Klare et al. 2007) (Table 10). No guidelines were available for MIC breakpoints for *W. confusa* and *W. paramesenteroides* and other antibiotic-bacteria combinations not indicated in Table 10.

4.3.3 Genotypic antibiotic resistance profiling

Antibiotic resistance genes were determined by a microarray hybridization assay (Perreten et al. 2005), and selected resistance genes were subsequently confirmed by PCR (chapter 3).

Table 10 Valid current guidelines for resistance breakpoints of particular species-antibiotic combinations

Antibiotics:	***Lb. fermentum***	***Lb. plantarum***	***Lc. lacits* subsp. *lactis***	***P. pentosaceus***	***P. acidilactici***	***St. infantarius* subsp. *infantarius* (microdilution)**	***St. infantarius* subsp. *infantarius* (disc diffusion)**
Ampicillin	4[a]	4[a]	4[a]	4[a]	4[a]	0.25[b]	24[b]
Chloramphenicol	4[a]	8[a]	8[a]	4[a]	4[a]	16[b]	17[b]
Clindamycin	4[a]	4[a]	4[a]	4[a]	4[a]	1[b]	15[b]
Erythromycin	4[a]	4[a]	4[a]	4[a]	4[a]	1[b]	21[b]
Fusidic acid	1[c]	IR[c]	n.a.	8[c]	8[c]	n.a.	n.a.
Gentamicin	8[a]	64[a]	8[a]	4[a]	4[a]	n.a.	n.a.
Kanamycin	16[a]	64[a]	8[a]	4[a]	4[a]	n.a.	n.a.
Linezoid	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	21[b]
Penicillin G	0.5[c]	2[c]	n.a.	1[c]	1[c]	0.12[b]	24[b]
Quinopristin / Dalfopristin	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	15[b]
Streptomycin	16[a]	64[a]	16[a]	4[a]	4[a]	n.a.	n.a.
Tetracycline	8[a]	32[a]	4[a]	4[a]	4[a]	8[b]	18[b]
Trimethoprim	8[a]	8[a]	IR[a]	8[a]	8[a]	n.a.	n.a.
Vancomycin	IR[c]	IR[c]	4[a]	IR[c]	IR[c]	1[b]	17[b]

[a] (European Commission 2005), [b] (CLSI 2006a), [c] (Klare et al. 2007) IR = intrinsic resistance, n.a. = no guidelines were available for this antibiotic-bacteria combination, n.t. = not tested; because antibiotics were not ready available.

4.4 Results

4.4.1 Phenotypic antibiotic resistance profiling

Phenotypic antibiotic resistance profiles were determined by disc diffusion and microdilution experiments. Both test approaches were used to compare resistance profiles and might relate defined inhibition zones to minimal inhibition concentration. Resistance breakpoints were based on guidelines (CLSI 2006a; European Commission 2005) if available (Table 10). Strains of tested species were susceptible against amoxicillin, amoxicillin/clavulanic acid, ampicillin, chloramphenicol, cloxacillin, erythromycin, fusidic acid, gentamycin, neomycin, penicillin G, and rifampicin Table 11. Tested species were also susceptible against clindamycin, except one *Lc. lactis* subsp. *lactis* strain (MIC of 8 µg/ml, no inhibition zone), against linezolid, except five strains of *P. pentosaceus* (MIC not tested, no inhibition zone), against mupirocin, except two strains of each pediococci (MIC not tested, no inhibition zone), against novobiocin, except one strain of *P. pentosaceus* (MIC not tested, no inhibition zone) and against quinopristin/dalfopristin, except two strains of *P. acidilactici,* four strains of *P. pentosaceus* and one strain of *St. infantarius* subsp. *infantarius* (MIC not tested, no inhibition zone).

Lc. lactis subsp. *lactis, W. paramesenteroides* and *St. infantarius* subsp. *infantarius* were susceptible against ciprofloxacin, both lactobacilli species against trimethoprim and *Lb. fermentum, P. acidilactici* and *Weissella* sp. against oxytetracycline and tetracycline.

Both lactobaclli and pediococci species displayed reduced susceptibility against ciprofloxacin, *W. confusa* and *W. paramesenteroides* against trimethoprim, trimethoprim/sulfamethoxazol and vancomycin and pediococci species against trimethoprim/sulfamethoxazol. Three *Lb. fermentum*, two *Lc. lactis* subsp. *lactis*, three *P. acidlicatici* and eleven *P. pentosaceus* (including the type strains of both pediocicci) were resistant against kanamycin (European Commission 2005). High MIC levels against kanamycin with up to 128 µg/ml were detected for some *Weissella* sp. and *St. infantarius* subsp. *infantarius* indicating resistant strains belonging to these species. Three *P. acidilactici* and one *P. pentosaceus* were trimethoprim resistant and four *Lc. lactis* subsp. *lactis*, four *P. acidilactici* and six *P. pentosaceus* (including the type strains of both pediocicci) were resistant against streptomycin (European Commission 2005). We detected 17% oxytetracycline and tetracycline resistance prevalence among non-*Enterococcus* LAB, with one *Lb. plantarum*, four *Lc. lactis* subsp. *lactis* and seven *St. infantarius* subsp. *infantarius. P.*

pentosaceus 9T-2211 displayed resistance to oxytetracycline but remained susceptible against tetracycline.

4.4.2 Genotypic antibiotic profiling

Twelve strains with phenotypic tetracycline resistance, one *Lc. lactis* subsp. *lactis* with phenotypic clindamycin resistance and the corresponding type strains were investigated on the presence of acquired resistance genes by microarray hybridization (Table 12). Detected antibiotic resistance genes were confirmed by PCR (chapter 3). The tetracycline resistance gene *tet*(S) was detected in all tetracycline resistant *St. infantarius* subsp. *infantarius* and in *Lc. lactis* subsp. *lactis*. *Lb. plantarum* 27FH harbored the tetracycline resistance gene *tet*(M). No resistance genes were found in the clindamycin resistant *Lc. lactis* subsp. *lactis* strain.

Table 11 Phenotypic antibiotic resistance profiles of *Lactobacillus fermentum*, *Lb. plantarum*, *Lactococcus lactis* subsp. *lactis*, *Pediococcus pentosaceus*, *P. acidilactici*, *Weissella paramesenteroides*, *W. confusa* and *Streptococcus infantarius* subsp. *infantarius* isolated from spontaneous *fènè* fermentation at small-scale and domestic production in Mali, determined by disc diffusion and microdilution experiments

First row of each antibiotic: Minimal inhibitory concentration (MIC) [µg/ml] of microdilution assays. Second row of each antibiotic: Diameter of the halo [mm] around the antibiotic disc; disc diameter of 6 mm.

Antibiotics (Antibiotic test range of microdilution experiments [µg/ml] | antibiotic disc concentration [µg])	**First row of each antibiotic: Minimal inhibitory concentration (MIC) [µg/ml] of microdilution assays Second row of each antibiotic: Diameter of the halo [mm] around the antibiotic disc; disc diameter of 6 mm**							
	***Lb. fermentum* (13)**	***Lb. plantarum* (9)**	***Lc. lacits* subsp. *lactis* (9)**	***P. pentosaceus* (23)**	***P. acidilactici* (4)**	***W. paramesenteroides* (3)**	***W. confusa* (16)**	***St. infantarius* subsp. *infantarius* (6)**
Amoxicillin / Clavulanic acid	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
(n.t. | 20/10)	25-34	29-43	25-37	22-32	24-27	30-38	22-38	15-37
Amoxicillin	≤0.125	≤0.125	≤0.125	0.125-0.5	0.125-0.5	≤0.125	≤0.125	≤0.125
(0.125-32 | n.t.)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Ampicillin	≤0.031-0.25	0.063-0.25	0.063-0.25	0.5-2	0.5-1	≤0.031-0.063	0.063-0.5	≤0.031-0.125
(0.031-64 | 10)	23-31	20-32	25-32	21-30	21-24	27-35	15-36	15-37
Chloramphenicol	1-2	1-2	0.25-2	≤0.125-1	0.25-2	0.5-2	2-4	1
(0.125-256 | 10)	21-25	21-27	22-30	23-31	25-28	20-27	20-31	21-30
Ciprofloxacin	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
(n.t. | 5)	6-9	6-7	10-23	6-10	6	11-13	6-23	14-26

Clindamycin	≤0.016	≤0.016-0.5	0.031-8	≤0.016	≤0.016	≤0.016-0.5	≤0.016-0.125	≤0.016
(0.016-32 | 2)	23-28	8-24	6-27	25-38	25-33	10-28	20-35	22-28
Cloxacillin	0.125-16	2-16	1-4	0.25-16	1-8	4-8	1-16	1-2
(0.031-64 | n.t.)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Erythromycin	≤0.016-0.25	0.063-0.125	0.063-0.25	≤0.016-0.125	≤0.016-0.125	≤0.016-0.063	0.031-0.063	≤0.016-0.031
(0.016-32 | 15)	23-28	23-29	22-30	22-32	24-28	21-31	21-34	15-32
Fusidic acid	≤0.016-0.063	0.063-2	0.125-1	≤0.016-0.125	≤0.016-0.125	0.125-1	0.25-0.5	0.5-2
(0.016-128 | 10)	22-27	12-24	13-21	20-27	19-24	10-22	15-28	17-28
Gentamycin	≤0.25-2	≤0.25-1	≤0.25-8	≤0.25-1	≤0.25-4	≤0.25-0.5	≤0.25-4	2-32
(0.25-64 | 10)	13-17	13.22	9-15	11-19	11-17	17-22	12-20	8-15
Kanamycin	4-32	2-16	1-32	0.5-64	≤0.5-128	2-16	8-128	64-128
(0.5-128 | 30)	8-11	8-17	8-20	6-12	6-10	11-15	6-16	6-14
Linezoid	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
(n.t. | 30)	21-30	24-28	20-30	6-34	28-31	25-36	23-35	19-32
Mupirocin	0.25-1	1-16	≤0.063-1	0.125-32	8	≤0.063-0.125	≤0.063-0.5	≤0.063
(0.063-128 | 200)	21-32	22-28	13-26	6-27	6-24	26-37	20-46	22-38
Nalidixic acid	>256	≥256	128-256	8-256	128-≥256	>256	32->256	>256
(0.125-256 | n.t.)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Neomycin	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
(n.t. | 30)	12-16	8-20	10-18	11-18	12-17	17-19	15-19	9-14
Novobiocin	0.125-1	≤0.031-0.063	0.063-0.25	0.5-32	≤0.031	0.031-0.25	0.25-2	0.063-1
(0.031-64 | 5)	13-21	17-26	12-21	6-25	26-32	15-25	15-26	21-27
Oxacillin	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
(n.t. | 1)	6	6-11	10-18	6	6	6	6-9	6-21
Oxytetracycline	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
(n.t. | 30)	14-21	6-21	10-26	6-23	17-23	15-25	16-29	6-27

Penicillin G	≤0.031-0.5	≤0.031-0.5	0.063-0.125	≤0.031-0.5	≤0.031-0.25	0.063-0.25	≤0.031-0.25	≤0.031-0.063
(0.031-64 | 10 units)	n.t	n.t.	22-33	23-31	26-31	22-28	21-39	18-37
Quinopristin / Dalfopristin	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
(n.t. | 15)	21-27	22-36	13-23	6-32	6-30	21-29	22-32	19-27
Rifampicin	≤0.125	≤0.125-0.25	0.5-16	≤0.125	≤0.125	0.25-2	1-8	≤0.125-0.25
(0.125-64 | 30)	25-34	21-30	14-26	24-36	28-35	17-24	16-28	15-34
Streptomycin	4-16	1-8	1-64	≤0.5-32	1-64	2-8	1-16	4-16
(0.5-256 | 10)	6-10	8-20	6-13	6-9	6-9	10-13	6-13	6-8
Tetracycline	≤1-2	2-32	≤1-32	≤1-2	≤1-2	≤1-2	≤1-2	8-32
(1-256 | 30)	19-23	6-22	6-16	16-22	17-26	19-28	20-33	6-28
Trimethoprim (Tri)	≤0.25-2	≤0.25-1	128-512	≤0.25-16	4-32	32-64	16-512	0.5-8
(0.25-512 | n.t.)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Tri / Sulfamethoxazol	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
(n.t. | 25)	6-15	12-29	6-16	6	6	6	6	17-30
Vancomycin	64-≥256	64->256	≤0.125-0.25	16-≥256	64-≥256	128-256	32-≥256	≤0.125-0.25
(0.125-256 | 30)	6	6	16-20	6	6	6	6	20-25

n.t. not tested because antibiotics were not ready available.

Table 12 Phenotypic and genotypic antibiotic resistance profiles of selected non-*Enterococcus* lactic acid bacteria determined by disc diffusion and microdilution tests and microarray hybridization, respectively

Strain	Phenotypic antibiotic resistance profile[1]		Resistance genes
	Disc diffusion[2] (diameter[mm])	Microdilution test MIC [µg/ml]	
Lb. plantarum DSM 20174 ^{T}	none	none	none
Lb. plantarum 27FH	T(6), TE(6)	TE (32)	*tet*(M)
Lc. lactis subsp. *lactis* DSM 20481^{T}	none	none	none
Lc. lactis subsp. *lactis* 5AU-2	S(6), TE(12), T(13)	TE (32)	*tet*(S)
Lc. lactis subsp. *lactis* 5AF	SXT(6), TE(10), T(11)	TE (16)	*tet*(S)
Lc. lactis subsp. *lactis* 8A-1	SXT(6), TE(9), T(10)	TE (16)	*tet*(S)
Lc. lactis subsp. *lactis* 13FR	TE(10), T(12)	TE (32)	*tet*(S)
Lc. lactis subsp. *lactis* 13DL-2112	CC(6)	CC (8)	none
St. bovis DSM 20480 ^{T}	S(6)	none	none
St. infantarius subsp. *infantarius* 3AG-1	S(6), TE(13), T(18)	TE (8)	*tet*(S)
St. infantarius subsp. *infantarius* 6BY-11b	K(6), SXT(6), TE(20), T(20)[3]	TE (16)	*tet*(S)
St. infantarius subsp. *infantarius* 8FD-1	K(6), S(6), TE(6), T(6)	TE (16)	*tet*(S)
St. infantarius subsp. *infantarius* 11FA-1	N(6), TE(16), T(19)	TE (16)	*tet*(S)
St. infantarius subsp. *infantarius* 13AY-1	FA(6), K(6), N(6) OX(6), S(6), TE(12), T(15)	TE (16)	*tet*(S)
St. infantarius subsp. *infantarius* 13CH	TE(14), T(18)	TE (16)	*tet*(S)
St. infantarius subsp. *infantarius* 13DC	K(6), S(6), TE(12), T(16)	TE (32)	*tet*(S)

[1] Abbreviations of antibiotics: CC, clindamycin; GM, gentamycin; K, kanamycin, OX, oxacillin; QD, quinopristin/dalfopristin; S, streptomycin; SXT, trimethoprim/sulfamethoxazol; T, tetracycline; TE, oxytetracycline.

[2] Disc diameter is 6 mm. [3] intermediate according to CLSI guidelines (CLSI 2006b). n.t.: Not tested.

4.5 Discussion

The FEEDAP Panel of the European Food Safety Authority (EFSA) suggests applying the Quality Presumption of Safety approach (QPS) for bacteria used in feed and food industries (Chamba and Jamet 2008). The QPS includes antibiotic resistance (AR) assessments of bacteria and states not to use strains carrying acquired antibiotic resistances as feed or food additives (European Commission 2005). Authorities have not yet established a consensus for antibiotic resistance testing of non-*Enterococcus* LAB (Flórez et al. 2008), taking into account varying results due to interference between media and antibiotics (Klare et al. 2005; Tosi et al. 2007) and different methodologies applied. We selected 84 non-*Enterococcus* LAB strains isolated from spontaneously *fènè* fermentation processes for AR profiling by disc diffusion and microdilution tests and subsequent genotypic profiling. We used both phenotypic tests to compare results and might to relate defined inhibition zones to exact MIC values. Phenotypic AR profiling by disc diffusion tests is less laborious and easier to perform than microdilution tests, which lead, however, to more accurate results. We observed for species-antibiotic combinations with higher MIC values, smaller halos around the antibiotic discs and vice versa, but could not relate a certain disc diameter to an exact MIC.

Bacteria with acquired antibiotic resistances present in food possess a risk for human health with so fare unknown dimensions. Overall 17% of the 84 strains isolated from spontaneous *fènè* fermentation processes were resistant to tetracycline and oxytetracycline. High seasonal-dependent oxytetracycline and tetracycline resistance levels were detected in enterococci isolated from the same environment (chapter 3). This tetracycline resistance prevalence may be related to the local farming practises, where oxytetracycline is most often used to treat mastitis (Bonfoh et al. 2003a). Many data on MIC values for *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Streptococcus* sp. were evaluated by E-test (Egervärn et al. 2007; Flórez et al. 2008; Hummel et al. 2007), by microdilution assays using other than the LSM or SSM media (Ge et al. 2007) or other methodology (Flórez et al. 2005). MIC data for the species *Lb. fermentum, Lb. plantarum, P. acidilactici* and *P. pentosaceus* of our study can be well compared with the study of Klare et al. (2007) on ten antibiotics. We found generally lower or similar MIC ranges for particular species-antibiotic combinations as previously published (Klare et al. 2007), except for *Lb. fermentum* 10HG-22, which was inhibited by erythromycin at 0.25 µg/ml. This value is one $\log_2$ step over the tentative

ECOFF value previously reported (Klare et al. 2007) and can be regarded as a outsider because one log_2-step difference correspond to normal test variation (Klare et al. 2007) and also due to we did not detect any acquired resistance gene in strain 10HG-22.

Little is so fare known about *W. confusa* and *W. paramesenteroides* in dairy products and to our knowledge we analysed the first time these species isolated from food, on their antibiotic susceptibility. *W. confusa* can cause severe infections in human (Svec et al. 2007; Vay et al. 2007). Phenotypic antibiotic resistance profiles on clinical isolates were recently published (Svec et al. 2007; Vay et al. 2007). MIC values of *W. confusa* strains isolated from spontaneous *fènè* fermentation processes were lower or identical to published MIC values (Svec et al. 2007; Vay et al. 2007), except for chloramphenicol, for which we detected with 2-4 µg/ml a one to two log_2 step higher MIC range as previously reported. A health concerning fact to consumer of *fènè* beside a general inadequate food safety is, that all analysed *St. infantarius* subsp. *infantarius* strains, isolated from spontaneous *fènè* fermentation, were resistant to oxytetracycline and tetracycline and harbored the *tet*(S) gene. *St. infantarius* subsp. *infantarius* is a member of the *St. bovis* / *St. equinus* complex (Schlegel et al. 2003) is an animal and human pathogen (Corredoira et al. 2008a; Corredoira et al. 2008b; Devriese et al. 1999). High tetracycline resistance prevalence of 65% for clinical *St. bovis* isolates has been reported (Streit et al. 2005). Tetracycline resistant *St. infantarius* subsp. *infantarius* in *fènè* may be due to sub-therapeutic oxytetracycline doses during mastitis treatments and were subsequently carried over to small-scale and domestic *fènè* production in the transporting containers for raw milk.

The *tet*(S) gene in *Lc. lactis* subsp. *lactis* and the *tet*(M) gene in *Lb. plantarum* have been found in tetracycline resistant isolates from food origin (Danielsen 2002; Wang et al. 2006). *Lc. garvieae* can no longer be discriminated from *Lc. lactis* subsp. on its clindamycin resistance (Walther et al. 2008), however clindamycin resistance in *Lc. lactis* subsp. has only been described in combination with erythromycin resistance triggered by the *erm*(B) gene (Walther et al. 2008). We confirmed the identification of the clindamycin resistant *Lc. lactis* subsp. *lactis* by sequencing of its 16S rRNA gene sequence after we could not detect any clindamycin resistance gene by micro-array hybridization. The antibiotic resistance genes *tet*(M), *tet*(L), *tet*(S), and *msrC* were detected in a previous work in enterococci from *fènè* microbiota (chapter 3). Tetracycline resistance genes are associated with mobile plasmids, transposoms and integrons and therefore highly transferable by conjugation (e.g. *tet*(S) associated to conjugative plasmids, *tet*(M)

associated to conjugative transposons) (Chopra and Roberts 2001). The potential health hazard of acquired antibiotic resistant genes lies in their transferability between different genera (Jay et al. 2005; Perreten et al. 1997; Teuber et al. 1999). Conjugative gene transfer is enhanced at high bacteria concentration and therefore the gastrointestinal tract of human and animal act as reservoir for antibiotic resistance genes (Ammor et al. 2007; Kastner et al. 2006), which might be transferred also to opportunistic pathogens. *Fènè* microbiota consists of a high bacterial concentration, representing a considerable antibiotic resistance gene pool, consumed by the rural population of Mali.

17% of 84 non-*Enterococcus* LAB selected for the development of safe adapted starter cultures for small-scale *fènè* production were resistant against tetracycline and oxytetracycline. This study suggests that in the environment we analyzed sub-therapeutic antibiotic pressure in animal husbandry selected for a tetracycline resistance gene pool, which is transferable between different genera at local conditions. We propose to address food safety, record local antibiotic purchase and maintain its correct application to improve consumer health in long term. A short-term measure would be to heat *fènè* prior to consumption or to introduce controlled fermentation by safe adapted starter cultures.

4.6 Acknowledgements

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5 Development and application of an adapted starter culture for sour milk production *(fènè)* at small-scale dairies in Mali

Data presented in this chapter will be submitted for publication to an international peer-reviewed journal in 2009 with the authorship:
Stephan Wullschleger, Leo Meile, Aminata Sissoko-Thiam, Bassirou Bonfoh, Sarah Baumgartner and Christophe Lacroix

5.1 Abstract

The complex microbiota of the Malian sour milk *fènè,* traditionally produced by spontaneous fermentation, was recently descriped. The putative human pathogen *Streptococcus infantarius* subsp. *infantarius* has now been identified as main acidifier in this microbiota. A novel developed starter consisting of *Lactobacillus fermentum*, *Lb. plantarum*, *Lactococcus lactis* subsp. *lactis* and *Weissella paramesenteroides* isolated from traditional microbiota combined with a commercial *St. thermophilus* culture displayed high acidification and stability during five back-slopping cultures at 35℃ and 40℃ and high antimicrobial activity to *Listeria ivanovii* HPB28 and *Enterococcus faecalis* DSM 2048^{T} at 30℃ and 35℃. This starter acidified fastest a t rural small-scale conditions (ambient temperature 27-36℃), reaching pH 4.8±0.2 compared to an industrial culture and traditional *fènè* fermentation with pH of 5.3±0.2 and 6.2±0.0, respectively within 9 hours. Post-heat treatment contamination was reduced but not eliminated compared to traditional fermentation. Standardized protocols are proposed to improve safety and quality in *fènè* production.

5.2 Introduction

Fermentation is one of the oldest of these practices, preserving foodstuffs for dry or winter seasons (Holzapfel 2002). Natural fermentation is often used to store food in single households and to extend shelf life in small-scale commercial production in regions where cooling systems are not easily available. Sour milks have the longest history in food preservation in Africa and have ensured dietary protein intake in the greater population (Caplice and Fitzgerald 1999).

Fènè, a spontaneously fermented dairy product traditionally produced by herdsmen carrying milk in their calabash while walking with the flock, is still produced by natural fermentation *i.e.*, without starter cultures, domestically and in a few low-tech small-scale dairies in Mali (Bonfoh et al. 2005a). Consumers prefer a consistent, creamy and sour (pH between 4.1 and 4.9) product. These characteristics are more easily attained during cold season according to local small-scale producers but quality and safety cannot be maintained and product inconsistencies may reflect fluctuations in microbial populations (chapter 2).

In a recent study the spontaneous microbiota of *fènè*, assayed during three seasons from single household and small-scale dairy fermentations, consisted of over 7.5±0.5

Log_{10}(cfu/ml) *Enterobacteriaceae* and 5.2±0.9 Log_{10}(cfu/ml) presumptive *Staphylococcus aureus*, indicating a human health hazard, which is corroborated with findings of enterococci and streptococci as dominant genera (chapter 2). In a total of 1583 presumable lactic acid bacteria (LAB) isolated from spontaneous *fènè* fermentations no *St. thermophilus* was detected but the animal and human pathogen *St. infantarius* subsp. *infantarius* was identified as dominant species. The uncontrolled use of antibiotics in animal husbandry led to antibiotic residues in milk (Bonfoh et al. 2003a) and induced antibiotic resistance in enterococci and non – *Enterococcus* LAB (chapter 3 and 4). In fact, all tested *St. infantarius* subsp. *infantarius* showed multiple phenotypic antibiotic resistances or harbored the tetracycline resistance gene *tet*(S) (chapter 4).
These represent pressing food safety issues since *fènè* is a staple for farmers and often consumed by rural and urban Malians alike. Traditional *fènè* production is moving from home-scale to small-scale production. The next developing step to medium-scale production requires however more control, consistency, qualtity and safety of the production process. We therefore characterized selected antibiotic susceptible LAB previous isolated from spontaneous *fènè* fermentation processes on their acidification properties, antimicrobial activity and exopolysaccharide (EPS) production. In a second step we developed adapted starter cultures for small-scale *fènè* production, which were subsequently field-tested at small-scale production in rural Mali.

5.3 Material and methods

5.3.1 Bacterial strains

In a previous study, 1583 presumable LAB were isolated during hot, cold and rainy seasons at domestic and small-scale *fènè* production sites and typed to strain level by a molecular approach (chapter 2). After antibiotic resistance profiling of non-*Enterococcus* LAB (chapter 4) three strains of *Weissella paramesenteroides*, one *Lactococcus lactis* subsp. *lactis*, two *Pediococcus acidilactici*, 17 *P. pentosaceus*, 13 *Lactobacillus fermentum* and eight *Lb. plantarum* were screened for different properties (acidification, antimicrobial activity and EPS production) for the development of an adapted starter culture for small-scale *fènè* production. *Streptococcus infantarius* subsp. *infantarius* was also isolated as dominant species from the *fènè* microbiota. Seven *St. infantarius* subsp. *infantarius* strains were selected to screen on functional features for evaluating their impact to spontaneous *fènè* fermentation processes. The type strains, *St. bovis* DSM

20480^T (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), *Lc. lactis* subsp. *lactis* DSM 20481^T, *W. paramesenteroides* DSM 20288^T, *P. pentosaceus* DSM 20336^T, *P. acidilactici* DSM 20284^T, *Lb. fermentum* DSM 20052^T, *Lb. plantarum* DSM 20174^T, *Enterococcus faecalis* DSM 2048^T, *Escherichia coli* JM109, and *Staphylococcus aureus* CAMP, (culture collection of the Laboratory of Food Biotechnology, ETH Zurich), and *St. infantarius* subsp. *infantarius* 8AG, previously isolated from a *fènè* fermentation process at small-scale level (chapter 2) were used for control of the screening experiments.

5.3.2 Microbiological analysis

The bacterial growth of single genera/species was recorded during successive back-slopping cultures, used for the development and evaluation of starter cultures, and experiments testing starter cultures at small-scale production in Mali. Single plate counts in two independent repetitions were used at plating conditions for: *Lc. lactis* subsp. *lactis* (M17 agar [Labo-life Sàrl, Pully, Switzerland] plates, 72 h, aerobic, 20°C), *W. paramesenteroides* (MRS [Labo-life Sàrl] -vancomycin [20 mg/l], 72 h, anaerobic, 20°C), *P. pentosaceus* (PSM-A (Simpson et al. 2006), 48 h, anaerobic, 37°C), lactobacilli (MRS pH 4.4, 48 h, anaerobic, 37°C) and *St. thermophilus* (M17, 24 h, aerobic, 43°C). Selectivity of the plating methods was checked with pure cultures of the different genera/species. Further plating was done for experiments testing starter cultures at small-scale production in Mali at the beginning of the fermentation, and after 9 (F9) and 22 h (F 22) on Violet Red Bile Dextrose (VRBD; VWR International AG, Dietikon, Switerland), aerobic, 37°C, 24 h for presumptive *Enterobacteriacea*; Baird Parker (BP; Labo-Life Sàrl), aerobic, 37°C, 24 h for presumptive *Staphylococcus aureus*; Azide Maltose agar (KF; Labo-Life Sàrl), aerobic, 43°C, 48 h for presumptive enterococci; and SM agar plates (Pacini et al. 2006) produced according to (chapter 6), aerobic, 43°C, 48 h for presumptive *St. bovis*.

5.3.3 Screening on functional properties

5.3.3.1 Acidification activity

The acidification activity of strains was screened in a microtiter assay. Strains were sub-cultured overnight first in MRS (20 g/l glucose) and secondly in MRS-Lactose (MRSL, 20 g/l lactose, Labo-Life Sàrl). The overnight cultures grown in MRSL were standardized with MRSL to OD_{600} = 0.1 (Uvikon 810P, Tegimenta AG, Rotkreuz, Switzerland) and 50

µl were centrifuged for five minutes at 10'000 rpm (Biofuge pico Heraeus, Kendro Laboratory products GmbH, Langenselbold, Germany). The cell pellet was resuspended in 250 µl milk prepared from skim milk powder (Oxoid AG, Pratteln, Switzerland) and transferred with a multi-channel pipette (Research pro, Eppendorf AG, Hamburg, Germany) into 96 well tissue culture test plates (Bioswisstec AG, Schaffhausen, Switzerland). *Lc. lactis* subsp. *lactis* and *W. paramesenteroides* were grown at 30℃, the other species at 37℃, aerobic. The pH was measured after 3, 6, 9, 15, 18 and 22 h to an accuracy of 0.2 pH units on pH indicator papers in the range from pH 3.8 to pH 5.4 and from pH 5.4 to pH 7.0 (VWR International AG, Dietikon, Switzerland). The experiment was performed twice.

The pH development during successive back-slopping cultures was recorded in four minutes intervals from two repetitions with the CINAC system (Alliance Instruments, Frepillon, France) and fermentation times were compared at pH 4.5 and pH 5.0 (Rault et al. 2007). The initial 2% inoculum, prepared by mixing equal amounts of each species, was added to 35 ml UHT milk (Migros) in 50 ml Falcon tubes (VWR International AG) and grown at 30℃, 35℃ and 40℃.

5.3.3.2 Antimicrobial activity

Supernatants of overnight cultures of strains grown in MRS were screened on antimicrobial activity against the target strains *Enterococcus faecalis* DSM 2048^T, *Escherichia coli* JM109, *Staphylococcus aureus* CAMP, *Listeria ivanovii* HPB 28 and *St. infantarius* subsp. *infantarius* 8AG by the agar well diffusion test (Turcotte et al. 2004). Antimicrobial activity of fermented milk supernatants from Cinac experiments were tested against *E. faecalis* DSM 2048^T and *L. ivanovii* HPB 28 and those from small-scale dairy fermentation in Mali against *L. ivanovii* HPB 28. Brain Heart Infusion (Becton Dickinson, Allschwil, Switzerland) soft agar containing 0.6% agar was used to grow the target strains overnight (18 h) at 37℃, except *L. ivanovii* HPB 28 which was cultivated in tryptic soy (Becton Dickinson) soft agar supplemented with 0.6% yeast extract overnight at 30℃. 80 µl of corresponding suppernatents were filled into 7 mm in diameter soft-agar wells prior to incubation.

5.3.3.3 Exopolysaccharide production

Semi-quantitative classification into high, medium and non-exopolysaccharide (EPS) producing strains was completed by combining the ropy feature of EPS producing strains

(Dierksen et al. 1997; Vescovo et al. 1989) with the fact that EPS protects colonies from colouration by ruthenium red (Stingele et al. 1996). Bacterial strains from overnight cultures grown in MRS were streaked onto MRSL agar plates supplemented with 0.08% ruthenium red, designated as MRSLr agar plates. White and strands forming colonies were regarded as high EPS producing strains; white no strands forming colonies as medium EPS producing strains and red colonies as non EPS producing strains.

5.3.4 Development of starter cultures

The pathogenic *St. infantarius* subsp. *infantarius*, originally present in *fènè* microbiota, was replaced for the development of adapted *fènè* starter cultures by a commercial EPS-producing *St. thermophilus* starter culture supplied by Danisco (Danisco, Brabrand, Denmark). Suitable strains previously screened on antibiotic susceptibility (chapter 4) were selected on fast acidification, antimicrobial activity against target strains, absence of antimicrobial activity among strains and EPS production in first development trial runs of a multi-species starter culture.

A second selection process of LAB for developing an adapted multi-species starter culture was applied. Nine strains of *P. pentosaceus*, three strains of *Lb. fermentum*, each one strain of *W. paramesenteroides* and *Lc. lactis* subsp. *lactis*, all isolated from the same spontaneous fermentation process at small-scale level during the cold season were chosen and combined to a basic *fènè* microbiota (BFM). A supplemented BFM (sBFM) was formulated with the BFM, a bacteriocinogenic *Lb. plantarum* also isolated from traditional *fènè* microbiota and the commercial *St. thermophilus* starter culture. The initial 2% inoculum, prepared by mixing equal amounts of each species, was added to 35 ml UHT milk (Migros) in 50 ml Falcon tubes (VWR International AG) and subsequent cultures were inoculated with 2% fermented milk of the previous culture. This was done during five subsequent back-slopping cultures in the Cinac system at 30℃, 35℃ and 40℃ in duplicate during two repetitions. The micro biota composition of the fifth cultures was regarde as a defined multi-species starter culture (DMS).

The pH development during successive back-slopping cultures was recorded in four minutes intervals from two repetitions with the CINAC system (Alliance Instruments, Frepillon, France) and fermentation times were compared at pH 4.5 and pH 5.0 (Rault et al. 2007).

5.3.5 Acidification capacity of *St. infantarius* subsp. *infantarius* in mixed cultures

The acidification capacity of *St. infantarius* subsp. *infantarius* was evaluated with three defined mixed starter cultures in triplicate: culture A consisting of the strains *St. infantarius* subsp. *infantarius* 11FA, *Lb. plantarum* 12BH and *Lc. lactis* subsp. *lactis* 8CM; culture B consisting of *St. thermophilus* DSM 20617^T, *Lb. plantarum* 12BH and *Lc. lactis* subsp. *lactis* 8CM; and culture C consisting of *Lb. plantarum* 12BH and *Lc. lactis* subsp. *lactis* 8CM. Single bacteria strains were grown overnight as described above and standardized to OD_{600} = 1 (Uvikon 810P) in MRSL. The inoculum of 200 µl was prepared as follows: an equal amount of each culture was mixed and centrifuged. The pellet was resuspended to the initial volume of 200 µl in UHT milk (Migros) and inoculated into 10 ml UHT milk in a 15 ml Falcon tube (Bioswisstec AG, Schaffhausen, Switzerland). Incubation was done at 37°C and pH (781 pH/Ion meter, Metrohm AG, Herisau, Switzerland) was recorded after 7 h and 24 h.

5.3.6 Adapted small-scale *fènè* production in Mali

Fènè production with an industrial *St. thermophilus* culture (Danisco, Kreuzlingen, Switzerland), the novel developed DMS, and traditional spontaneous fermentation with endogenous bacteria were tested at small-scale production in the low-tech dairy Bagan Yiri Wa Ton in Kasséla, a village 40 km from the capital city Bamako (Mali). *Fènè* was produced at 5 l batches in new metallic vats disinfected with bleach (sodium hypochlorite) rinsed with hot water (90°C) and covered by a metallic lid (still aerobic conditions) and in traditional used plastic vats covered by a mosquito net. Heated (on fire or in a batch gas pasteurisator to 85°C) cow milk was cooled at ambient temperatures (32.7±3.0°C) to 42°C before milks were inoculated with 2% starter culture.

The defined multi-species starter culture was produced as fresh culture at the Laboratoire Central Vétérinaire (LCV, Bamako, Mali) from frozen (–70°C) stock cultures in 30% glycerol. Stock cultures were first sub-cultured overnight in MRS and secondly in MRSL before 200 ml UHT whole milk (Azar, Bamako, Mali) were inoculated with 1.5 ml MRSL cultures and incubated for 48 h fermentation at 30°C for *Lc. lactis* subsp. *lactis* and *W. paramesenteroides* and at 37°C for lactobacilli. Species grown in UHT milk were mixed in equal amounts for preparing the initial inoculum for *fènè* production. Industrial lyophilized *St. thermophilus* culture was dissolved in 500 ml UHT milk, aliquoted in 5 ml into 15 ml Falcon tubes and stored frozen at -20°C until use. This culture was either

used for direct inoculation of milk or first cultured overnight in 200 ml UHT milk at 37℃ and mixed to the developed the DMS.

Traditional *fènè* was produced in 5 l batches previously described (chapter 2). Samples were taken at the beginning of the fermentation (F0), after 9 (F9), and 22 h (F22). Bacterial growth, pH (mobile pH-meter 604, Metrohm AG) and ambient and milk temperatures were determined for F0, F9 and F22 and antimicrobial activity was tested in the samples F22 against *L. invanovii* HBP28.

Recontamination of heat-treated milk by opportunistic pathogens during the tested fermentation processes was investigated by the presence of *Enterobacteriaceae*, *S. aureus*, enterococci and *St. bovis* in fermented milk microbiota. Experiments were repeated twice in a 14 days interval.

5.3.7 Statistical analysis

Differences in bacterial counts [Log_{10}(cfu/ml)], antimicrobial activity and fermentation times were tested for statistical significance (p=0.05) with Turkey's HSD-test or the independent t-test using SPSS software (version 16.0 SPSS, Chicago, IL, USA).

5.4 Results

5.4.1 Screening on functional properties

5.4.1.1 Acidification properties

Rapid acidification is a priority for development of starter cultures suitable for sour milk production. Mean acidification data measured in the microtiter assay for the 51 strains selected for the development of starter cultures are shown by species in Table 13. Strains within a species generally exhibited similar acidification activities in milk; this is seen by the low standard deviations of pH. *St. infantarius* subsp. *infantarius* displayed the fastest acidification rate with a pH of 4.2±0.2 after 9 hours and the lowest pH of 3.9 after 22 hours (Table 13). Good acidification properties were also detected for *W. paramesenteroides* with pH 4.8±0.1 after 22 h. Strains of the other species did not decrease pH below 5.0 within 22 h.

Table 13 Acidification properties in single strain fermentation over 22 h of all strains per species, which were selected for the development of a defined multi-species starter culture and *St. infantarius* subsp. *infantarius*. Screening experiments were done in duplicate

	Number of strains	3 h	6 h	9 h	15 h	18 h	22 h
St. infantarius subsp. *infantarius*	7	5.6±0.4^{a}	4.8±0.2^{a}	4.2±0.2^{a}	4.1±0.3^{a}	4.0±0.2^{a}	3.9±0.1^{a}
Lc. lactis subsp. *lactis*	1	6.7±0.0^{d}	6.6±0.0^{e}	6.5±0.0^{d}	6.4±0.1^{d}	6.3±0.0^{d}	6.3±0.0^{e}
W. paramesenteroides	3	6.4±0.0^{c,d}	6.2±0.1^{d}	5.7±0.3^{b}	5.6±0.2^{b}	5.3±0.3^{b}	4.8±0.1^{b}
P. pentosaceus	17	6.2±0.1^{b,c}	6.0±0.2^{b,c}	5.8±0.2^{b}	5.4±0.4^{b}	5.2±0.6^{b}	5.0±0.6^{b,c}
P. acidilactici	2	6.4±0.2^{c,d}	6.2±0.1^{c,d}	6.0±0.2^{b,c}	5.9±0.2^{b,c}	5.7±0.3^{b,c}	5.6±0.4^{c,d}
Lb. fermentum	13	6.1±0.1^{b}	5.9±0.2^{b}	5.9±0.2^{b}	5.7±0.1^{b}	5.6±0.1^{b,c}	5.5±0.2^{c,d}
Lb. plantarum	7	6.5±0.0^{d}	6.4±0.0^{d,e}	6.3±0.1^{c,d}	6.2±0.1^{c,d}	6.1±0.1^{c,d}	6.0±0.2^{d,e}

^{a,b,c,d,e} Values with different letters in a column are significantly different by Tukey's test (p=0.05).

5.4.1.2 Antimicrobial activity

Antimicrobial activity was detected in three strains: *St. infantarius* subsp. *infantarius* 3AG, *St. infantarius* subsp. *infantarius* 6C and *Lb. plantarum* 12BH. The antimicrobial activity of *Lb. plantarum* 12BH was not affected by pH adjustment to pH 6.0 and heat treatment to 121°C for 15 minutes, whereas antimicr obial activity of *St. infantarius* subsp. *infantarius* 6C was affected by heat treatment but not by pH adjustment. Digestion with proteinase K degraded antimicrobial activity of the supernatants of both *St. infantarius* subsp. *infantarius* 6C and *Lb. plantarum* 12BH. *Lb. plantarum* 12BH is therefore assumed to produce heat-stable and *St. infantarius* subsp. *infantarius* 6C a heat-sensible bacteriocin-like inhibitory compounds. The antimicrobial activity of *St. infantarius* subsp. *infantarius* 3AG was not further analysed due to its limited activity against only *St. infantarius* subsp. *infantarius* 8AG displaying a maximum inhibition diameter of 9 mm (well diameter 6 mm). *St. infantarius* subsp. *infantarius* 6C formed a maximum inhibition diameter against *St. infantarius* subsp. *infantarius* 8AG of 12 mm and an average inhibition diameter of 12.0±2.8 mm to *L. ivanovii* HBP28. Highest antimicrobial activity was observed for strain *Lb. plantarum* 12BH, with 18.7±0.6 mm to *L. ivanovii* HBP28 and a maximum diameter of 11 mm to *E. faecalis* DSM 2048^{T}.

5.4.1.3 Exopolysaccharide production

94% of the 51 strains tested were medium EPS producer, appearing in white colonies. White strands forming colonies were detected for two strains *St. infantarius* subsp. *infantarius* 6BY-11b and *P. acidilactici* 10GK, whereas *Lc. lactis* subsp. *lactis* 8CM was the sole strain forming red colonies. The commercial EPS-producing *St. thermophilus* starter culture showed clearly higher EPS-production as the strain *P. acidilactici* 10GK investigated by visual observation when grown in UHT-milk.

5.4.2 Development of adapted starter cultures for small-scale *fènè* production

Two starter cultures were developed: a starter culture consisting of the commercial *St. thermophilus* starter culture and the bacteriocinogenic *Lb. plantarum* 12BH (D2S) developed through the first approach applied and a defined multi-species starter culture (DMS) consisting of the five species *St. thermophilus*, *Lb. plantarum*, *Lb. fermentum*, *W. paramesenteroides* and *Lc. lactis* subsp. *lactis*. The DMS was developed by five subsequent back-slopping cultures from the supplemented basic *fènè* microbiota

(sBFM). The D2S, the sBFM and the industrial *St. thermophilus* starter culture were compared to each other during five subsequent back-slopping cultures at 30℃, 35℃ and 40℃ in the Cinac system.

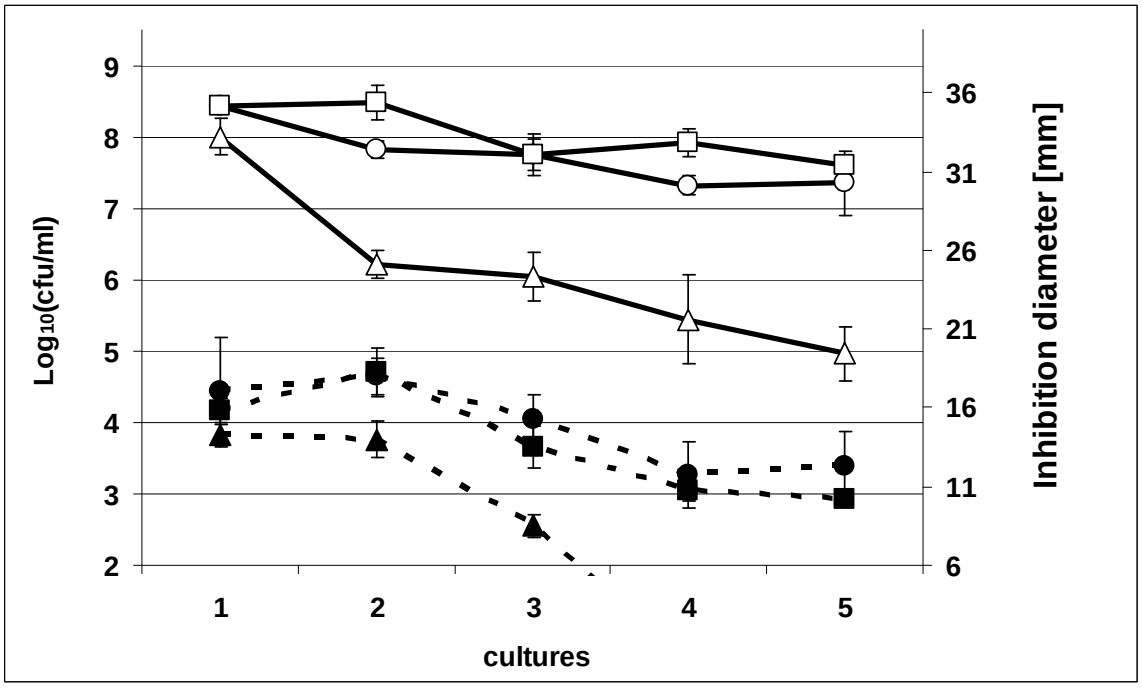

Figure 7 Development of bacterial counts during five subsequent back-slopping cultures at 30℃, 35℃ and 40℃ of the bacteriocinogenic *Lb. plantarum* 12BH used in the defined two-species starter culture (D2S) and corresponding antimicrobial activity of D2S to *L. ivanovii* HBP28.
Means and standard deviations were calculated from four replications.
○ Bacterial counts (black line) at 30℃; □ at 35℃ and Δ at 40℃.
● Antimicrobial activity (dashed line) to *L. ivanovii* at 30℃; at ■ 35℃ and at ▲ 40℃.

Bacterial counts of the commercial *St. thermophilus* starter culture, used in the D2S, sBFM and as original starter culture, were between 8.8±0.3 Log_{10}(cfu/ml) and 9.2±0.2 Log_{10}(cfu/ml) and very stable in all cultures and temperatures (results not shown). Bacterial counts for *Lb. plantarum* 12BH in the D2S decreased steadily from culture to culture regardless of temperatures (Figure 7). Lactobacilli counts, i.e. *Lb. plantarum* and *Lb. fermentum* in the sBFM, were stable with counts from 7.9±0.2 Log_{10}(cfu/ml) to 7.2±0.1 Log_{10}(cfu/ml) from the second to the fifth culture and were significantly higher in the first culture with 8.4±0.1 Log_{10}(cfu/ml) to 8.0±0.2 Log_{10}(cfu/ml) at all temperatures (result not shown). Highest lactobacilli counts were found at 35℃, except in the fifth culture where no counts differed among temperatures (results not shown). Bacterial counts of *Lc. lactis* subsp. *lactis* were with over 7.1±0.1 Log_{10}(cfu/ml) significantly higher

throughout all cultures at 30℃ than at 40℃ (Figur e 8A). Lactococci counts constantly decreased to 6.1±0.1 Log_{10}(cfu/ml) and 4.8±0.3 Log_{10}(cfu/ml) in the fifth cycle at 35℃ and 40℃, respectively (Figure 8A). Bacterial count s of *W. paramesenteroides* decreased significantly from culture to culture at all temperatures, except at 30℃ where stable counts of 6.0±0.1 Log_{10}(cfu/ml) were reached after the fourth culture (Figure 8B). The applied fermentative environment was not beneficial for *P. pentosaceus*, which could not be detected in high numbers after the third culture at 30℃, 35℃ and 40℃ (Figure 8C).

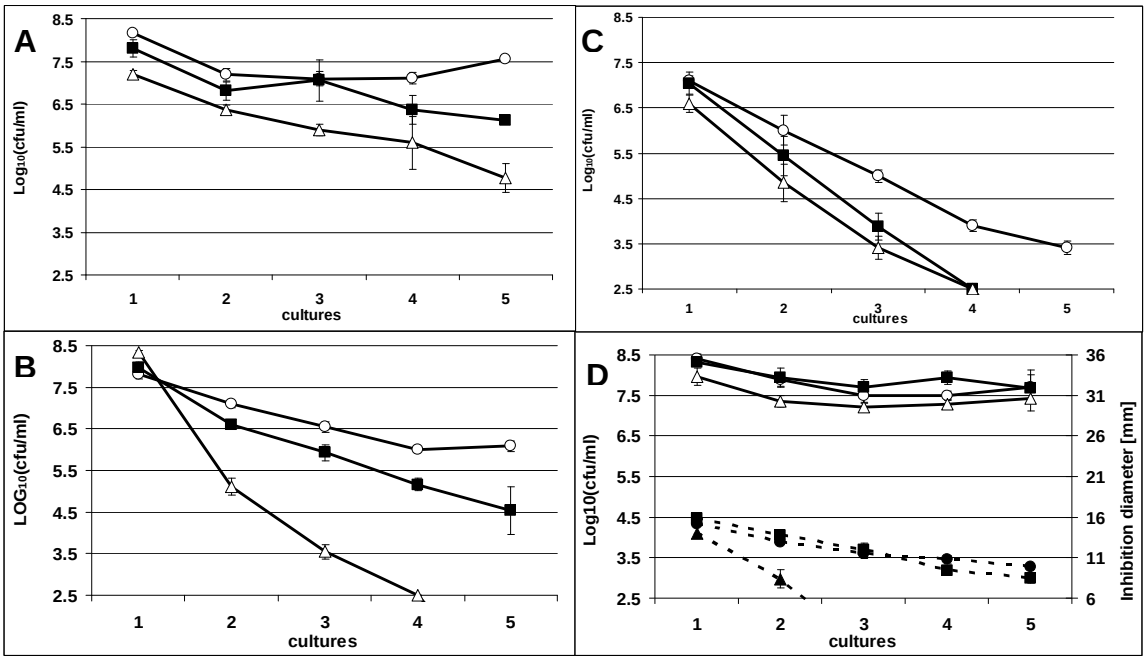

Figure 8 Development of bacterial counts and antimictobial activity to *L. invanovii* HPB 28 (D) during five back-slopping cultures at 30℃, 35℃ an d 40℃ of (A) *Lc. lactis* subsp. *lactis*, (B) *W. paramesenteroides*, (C) *P. pentosaceus*, (D) lactobacilli present in the supplemented basic *fènè* microflora.

Means and standard deviations were calculated from four replications. ○ Bacterial counts (black lines) at 30℃; ■ at 35℃ and ∆ at 40℃ ● Antimicrobial activity (dash lines) to *L. ivanovii* at 30℃; at ■ 35℃ and at ▲ 40℃.

Antimicrobial activity of strain *Lb. plantarum* 12BH could be maintained best at 30℃ and 35℃ in the D2S and in the sBFM as shown in Figure 7 and 8D, respectively. *L. ivanovii* HPB28 was more inhibited than *E. faecalis* DSM 2048^T by the presence of *Lb. plantarum* 12BH. *E. faecalis* DSM 2048^T was not inhibited by the sBFM after the second and by the D2S after the third culture (results not shown). Antimicrobial activity decreased

concurrently with bacterial counts of *Lb. plantarum* 12BH form culture to culture and was significantly higher after the primary culture at 30℃ than at 40℃ (see *L. ivanovii* in Figure 7).

The fermentation time required reaching pH 4.5 and 5.0 at 35℃ and 40℃ displayed smaller standard deviations than those times recorded at 30℃. During the first back-slopping cultures, the sBFM showed significantly shorter fermentation times to reach pH 4.5 with 332±44 min and 490±34 min at 40℃ and 35℃ , respectively than the commercial *St. thermophilus* culture with 450±63 min and 728±18 min at 40℃ and 35℃, respectively. The D2S showed intermediate fermentation times to the other two starters. No significant differences of fermentation times between the three starter cultures were found in the subsequent back-slopping cultures (Figure 9A-C).

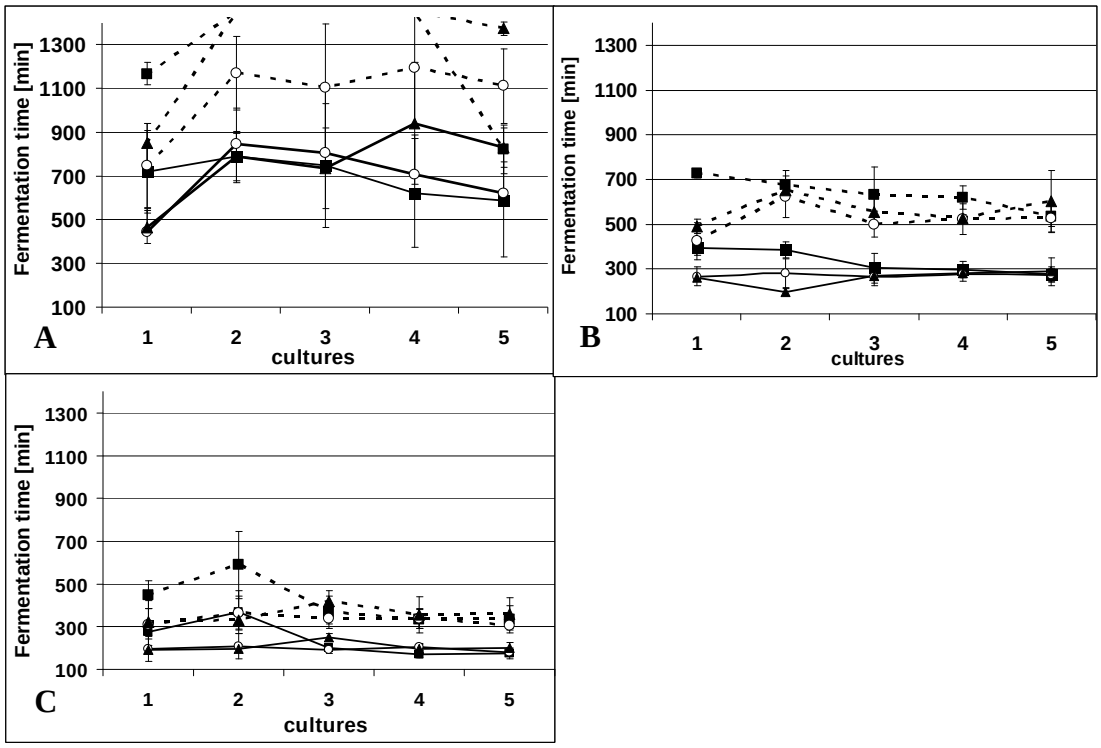

Figure 9 Fermentation times to reach pH 5.0 (black line) and pH 4.5 (dashed black line) by the supplemented basic *fènè* microflora (▲), the defined two-species starter culture (D2S) (○) and the industrial *St. thermophilus* starter culture (■) during five subsequent back-slopping cultures at 30℃ (A), 35℃ (B) and 40℃ (C).

Means and standard deviations were calculated from four replications.

5.4.3 Acidification capacity of *St. infantarius* subsp. *infantarius* in mixed cultures

The acidification capacity of *St. infantarius* subsp. *infantarius* was analyzed in three mixed cultures. The three cultures A, B and C were inoculated with 6.5 ± 0.0 Log_{10}(cfu/ml) streptococci, 7.2 ± 0.1 Log_{10}(cfu/ml) lactococci and 5.0 ± 0.1 Log_{10}(cfu/ml) lactobacilli. Fastest acidification was detected for culture A and culture B with a pH of 5.3 ± 0.3 and 5.2 ± 0.1, respectively after 7 h. Culture A reached pH 4.1 ± 0.1 and culture B 4.5 ± 0.0 ($p = 0.03$) after 24 h. Culture C consisting only of *Lc. lactis* subsp*. lactis* 8CM and *Lb. plantarum* 12BH only reached pH 6.5 ± 0.0 and pH 6.2 ± 0.0 after 7 h and 24 h, respectively.

5.4.4 Adapted small-scale *fènè* production in Mali

Fermentation trails were performed with simple conditions representing local production; without heat control of the fermentation process at the end of the rainy season (August and September 2008) at ambient temperatures between 27-36℃. Fermentation processes of milk inoculated with the industrial *St. thermophilus* starter culture were started with 6.7 ± 0.9 Log_{10}(cfu/ml) *St. thermophilus*. Fermentation processes of milk inoculated with the DMS started with bacterial counts for *St. thermophilus*, lactobacilli, *W. paramesenteroides* and *Lc. lactis* subsp. *lactis* of 7.2 ± 0.9 Log_{10}(cfu/ml), 6.0 ± 0.4 Log_{10}(cfu/ml), 5.6 ± 0.4 Log_{10}(cfu/ml) and 5.5 ± 0.1 Log_{10}(cfu/ml), respectively (Table 14). Milk cooled to 30.0±3.1℃ within the first three ho urs of the fermentation process after inoculation. The pH decreased most rapidly in milk inoculated with the DMS, followed by the commercial *St. thermophilus* starter and finally milk traditionally [spontaneously] fermented without starter culture with pH of 4.8±0.2, 5.3±0.2 and 6.2±0.0, respectively after 9 h of the fermentation process. The pH of milk inoculated with the DMS was significantly lower with 4.5±0.2 as pH of traditional fermented milk with 4.9±0.0 after 22 h of the fermentation process, whereas pH of milk fermented with the industrial *St. thermophilus* starter culture was intermediate with 4.7±0.2. No differences in pH decrease of milks fermented with starter cultures in plastic or metallic vats were detected.

Significant bacterial growth was determined for all bacterial species inoculated as starter cultures during the fermentation process with 9.5 ± 0.8 Log_{10}(cfu/ml) for *St. thermophilus* of the industrial starter and 9.7 ± 0.8 Log_{10}(cfu/ml), 7.3 ± 0.4 Log_{10}(cfu/ml), 7.8 ± 0.6 Log_{10}(cfu/ml) and 7.6 ± 0.4 Log_{10}(cfu/ml) for *St. thermophilus*, lactobacilli, *Lc. lactis*

subsp. *lactis*, and *W. paramesenteroides*, respectively of the DMS in the final products after 22 h fermentation (Table 14). Antimicrobial activity of the DMS to *L. ivanovii* was retained at small-scale fermentation showing halos between 8 and 11 mm with the agar diffiusion test.

Post heat treatment contamination by opportunistic pathogens of milks fermented with starter cultures was reduced by around two Log_{10} steps compared to traditional spontaneous fermentation. *St. bovis* counts were significantly reduced from 6.2 ± 0.1 Log_{10}(cfu/ml) in traditional produced *fènè* to 3.4 ± 0.6 Log_{10}(cfu/ml) in *fènè* produced with starter cultures in the traditional plastic vats (Table 15). No differences on bacterial counts were determined between the use of plastic or metallic fermentation vats (Table 15).

Table 14 Bacterial counts of starter microorganisms of the industrial *St. thermophilus* starter and the defined multi-species starter culture applied for small-scale *fènè* fermentations in rural Mali at inoculation, 9 h and 22 h of the fermentation process at ambient temperatures (30.0±3.1℃)

	Industrial Starter	**Defined multi-species starter culture**			
	St. thermophilus	***St. thermophilus***	**Lactobaccilli**	***Lc. lactis* subsp. *lactis***	***W. paramesenteroides***
Inoculation	6.7±0.9 [a]	7.2±0.9 [a]	6.0±0.4 [a]	5.6±0.4 [a]	5.5±0.1 [a]
9 h	8.6±0.7 [b]	8.4±0.4 [a]	6.8±0.8 [a,b]	7.1±0.4 [b]	6.6±0.8 [a,b]
22 h	9.5±0.8 [b]	9.7±0.8 [b]	7.3±0.4 [b]	7.8±0.6 [b]	7.6±0.4 [b]

[a,b] Values with different letters in a column are significantly different by Tukey's test (p=0.05).

Table 15 Post heat treatment contamination by opportunistic human pathogens [Log_{10}(cfu/ml)] in *fènè* produced by starter cultures in metallic or plastic vats or traditional fermentation

	Pasteurized milk		**9 h of fermentation**		**22 h of fermentation**		
Fermentation vat	**Metallic**	**Plastic**	**Metallic**	**Plastic**	**Metallic**	**Plastic**	***Fènè***
Staphylococci	n.d.	1.3±0.2	2.8±1.6	2.4±0.8	2.4±1.3	2.9±1.6	3.4±1.7
Enterobacteriaceae	n.d.	n.d.	4.0±0.6	2.3±1.0	4.8±0.5	3.8±1.4	5.3±0.6
Enterococci	1.3**	2.0±0.4	4.4±1.3	3.2±1.7	4.0±1.8	3.5±1.7	6.6±0.6
St. bovis-group	2.6±0.4	2.9±0.5	4.1 ±1.2	3.5±0.6	3.8±1.0 [a]	3.4±0.6 [a]	6.2±0.1 [b]

n.d. = not detected at the detection limit of 10 cfu/ml.

** Only detected in one repetition.

[a,b] Values with different letters are significantly different by Tukey's test (p=0.05).

5.5 Discussion

The microbiota of several indigenous fermented milk products was described in recent years (Abdelgadir et al. 2001; Beukes et al. 2001; Dewan and Tamang 2007; Mathara et al. 2004; Obodai and Dodd 2006; Zamfir et al. 2006; Zhang et al. 2008) and some isolated bacteria were further characterized on functional or even probiotic properties (Ayad et al. 2004; Jiwoua Ngounou et al. 2003; Mathara et al. 2008; Olasupo et al. 2001; Patrignani et al. 2006). The discovery of the environmental contaminant and putative human and animal pathogen *St. infantarius* subsp. *infantarius* (Corredoira et al. 2008a; Corredoira et al. 2008b; Devriese et al. 1999; Stone 2004; zur Hausen 2006), dominating (chapter 2) and leading acidifier (this study) in spontaneous *fènè* fermentation, the high prevalence of acquired tetracycline resistance in these strains (chapter 4) as well as the presence of bacteria belonging to the *St. bovis* / *St. equinus* complex (Schlegel et al. 2003) in other indigenous African sour milks (Abdelgadir et al. 2008; Gonfa et al. 2001; Isono et al. 1994) strongly supports the development of safe adapted starter cultures for indigenous fermented milks.

A further very important aspect is that controlled fermentation process for local food production stimulate the agro industry of developing countries (Rolle and Satin 2002). This not only because product quality and safety are improved but also due too product shelf-live is extended, the fermentation process is scalable, economical and environmenttal friendly (Rolle and Satin 2002). Small or medium scale enterprises are more appropriated for developing countries because they need low capital investments, require only semi-outomated technologies which are easier to maintain locally and lead to more employement (Benkerroum and Tamime 2004). The realization of such medium-scale enterprises needs minimal technological improvements such as pasteurization, microbiological sound developed starter cultures, mechanization of key processing steps, appropriate conditioning, packaging and labelling as well as governmental legislation and control guaranteeing product quality and safety (Benkerroum and Tamime 2004). Furthermore quality and safety measures (HACCP), improved logistics, education of producers and sustainable product marketing, without neglecting cultural background and current local infrastructure has to be set-up to successfully introduce medium-scale fermentation processes (Amoa-Awua et al. 2007; Benkerroum and Tamime 2004; Bonfoh et al. 2006; Holzapfel 1997; Holzapfel 2002; Ijabadeniyi and Omoya 2006; Jay et al. 2005; Kebede et al. 2007; Motarjemi 2002; Rolle and Satin 2002).

Lc. lactis subsp. *lactis*, *W. paramesenteroides*, *P. pentosaceus*, *P. acidilactici*, *Lb. fermentum* and *Lb. plantarum* strains, isolated from spontaneous *fènè* fermentation processes, previously screened for antibiotic resistance (chapter 4) were investigated in this study for acidification properties, antimicrobial activity and EPS production. The stability of a starter culture was evaluated on constant bacterial counts, fermentation times and functional features. A multi-strain and multi-species starter culture is better adapted to changing production conditions (Teuber 1995) usually found in non-controlled spontaneous fermentation processes, such as at small-scale dairies in Mali. The stability of the sBFM and D2S within five successive back-slopping cultures at 30℃, 35℃ and 40℃ was compared to an industrial *St. thermophilus* starter culture. Stable bacterial counts, indicating species' importance for the fermentation process, were detected for *St. thermophilus* and lactobacilli at all temperatures, lactococci at 30℃ and 35℃ and *W. paramesenteroides* at 30℃ in the sBFM. *P. pentosaceus* could not be found in high numbers after three back-slopping cultures in the sBFM and was therefore not used in the defined multi-species starter culture (DMS) tested at small-scale production in Mali.

Starter cultures with protective features increase product shelf-life and improve food safety especially in places were adequate cooling is difficult to maintain. Benkerroum et al. (2000) reported reduced cell counts of *L. monocytogenes* and an increased shelf-life if a bacteriocin producing *Lc. lactis* strain was included in the production of the Moroccan cheese jben. The *Lb. plantarum* strain (12BH), inhibiting growth of *L. ivanovii* HPB28 and *E. faecalis* DSM 2048^{T} by a heat-stable bacteriocin like inhibitory substance, was successfully integrated into the DMS.

Exo-polysaccharid (EPS) production is a desirable feature of bacteria applied in dairy products because EPS act as natural biothickener leading to higher consistency and viscosity of the product and reduced syneresis (Ruas-Madiedo and de los Reyes-Gavilan 2005). We combined the ropy feature of EPS forming colonies and the capacity of EPS to protect colonies from colouration by ruthenium red to a novel screening methodology. Following this approach, we only found two high EPS producing strains, whereas 94% of the strains were classified as medium EPS producers. This approach might be regarded as not very sensitive unless it would first be validated by quantification of EPS produced.

Consumers of *fènè* ask for a consistence and creamy product, we therefore selected a high EPS producing *St. thermophilus* culture for the DMS. Preliminary sensorial testing with local consumers attributed to the *fènè* produced with the DMS a high consumer

acceptance although taste is different to traditional *fènè*. In contrast, *fènè* produced with the commercial *St. thermophilus* culture displayed too ropy features for local consumers.

Post heat treatment contamination of milk fermented with starter cultures by opportunistic pathogens could be reduced but not eliminated at small-scale production at ambient temperatures in rural Mali. This indicates the pressing need for more standardized production with temperature controlled fermentation and rigorous hygiene measures.

We also pointed out that the human and animal pathogen *St. infantarius* subsp. *infantarius* is the main acidifier in spontaneous *fènè* fermentation and should not be used in a starter culture due to its pathogenicy reasons in human medicine. But their could also be the relevance that *St. infantarius* subsp. *infantarius* isolated from *fènè* microbiota is adapted to milk and lost its pathogenicy features comparable to *St. thermophilus* (Delorme 2008). Recently, it has been proposed to apply *St. gallolyticus* subsp. *macedonicus* as adjunct starter for dairy fermentations (De Vuyst and Tsakalidou 2008) although it belongs to the SBSEC (Schlegel et al. 2003).

5.6 Conclusion

We developed by sound microbiological and biotechnological methodologies a multi-species starter culture, which is adapted to small-scale sour milk production in rural African low-tech dairies with bacteria isolated from the spontaneous *fènè* microbiota. Its application and preliminary sensorial testing on-site in Mali were positive and may now open in the future the possibility for local producer to transform from small-scale to medium-scale production.

5.7 Acknowledgement

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6 Molecular typing and antibiotic resistance profiling of *Streptococcus infantarius* subsp. *infantarius* and *Streptococcus gallolyticus* subsp. *macedonicus*, isolated from spontaneously fermented African sour milk products

Data presented in this chapter will be submitted for publication to an international peer-reviewed journal in 2009 with the authorship:

Catrina Niklaus, Stephan Wullschleger, Christoph Jans, Vincent Perreten, Christophe Lacroix, Bassirou Bonfoh and Leo Meile

6.1 Abstract

Spontaneously fermented milks are principle protein sources in the diet of rural Africans. Fermentative flora of these milks contains the animal and human pathogen *Streptococcus infantarius* subsp. *infantarius*. Raw and sour milk samples from Kenya, Mali and Somalia were investigated detecting and identifying bacteria belonging to the *St. bovis* / *St. equinus* complex (SBSEC). The phenotypic and genotypic antibiotic resistance profiles of selected isolates were analyzed by microdilution and microchip-hybridization, respectively. 95 *St. infantarius* subsp. *infantarius* and 26 *St. gallolyticus* subsp. *macedonicus* were isolated from 29 samples and identified by PCR, RFLP and rep-PCR to strain level. Cell count comparison on *St. macedonicus* (SM) semi-differential medium and SM tetracycline enriched medium resulted in a maximum of 98.8% and a median of 11.8% tetracycline resistant bacteria. 13 of 20 selected SBSEC bacteria isolated from different products displayed resistance to tetracycline and harbored either the *tet*(M), *tet*(L) or *tet*(S) gene. Five of these strains displayed multidrug resistance against ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, neomycin, penicillin, streptomycin and tetracycline and only five strains were susceptible to all 11 tested antibiotics. Combined erythromycin and tetracycline resistance of two *St. gallolyticus* subsp. *macedonicus* strains was related to the *erm*(B) and the *tet*(M) genes. This study demonstrates the existence of an elevated antibiotic resistance gene pool among opportunistic pathogens in African sour milks and raises the question if identical strains can be isolated from sour milks and human bacteremia or if strains of the SBSEC are adapting to milk fermentation displaying *St. thermophilus*-like properties.

6.2 Key words

Streptococcus bovis, *Streptococcus infantarius* subsp. *infantarius*, *Streptococcus gallolyticus* subsp. *macedonicus*, antibiotic resistance, tetracycline, Africa, sour milk

6.3 Introduction

Streptococcus infantarius subsp. *infantarius* was recently reported in the microbiota of the fermented camel milk *Gariss* from Sudan (Abdelgadir et al. 2008) and in the soured milk *fènè* from Mali (chapter 2) both at > 10^8 cfu/ml. These artisanal products are a principle protein source in the diet of rural populations of Africa (Abdelgadir et al. 2008) (chapter 2) where *St. infantarius* subsp. *infantarius* is consumed in high numbers.

St. infantarius subsp. *infantarius* is a member of the *St. bovis* / *St. equinus* complex (SBSEC). Bacteria of the SBSEC are present in normal human colons (Noble 1978) and livestock animals (Farrow et al. 1984). A strong relation between bacteremia or endocartitis caused by *St. infantarius* subsp. *infantarius* and other SBSEC bacteria and colonic tumors and non-colonic cancer has been reported (Corredoira et al. 2008a; Corredoira et al. 2008b).

Clinical isolates of SBSEC historically classified into biotype I, biotype II/1 and II/2 (Coykendall 1989) have been reclassified into four DNA homology clusters incorporating *St. macedonicus* as *St. gallolyticus* subsp. *macedonicus* and *St. alactolyticus* (Schlegel et al. 2003). *St. gallolyticus* subsp. *macedonicus* is described as a food-grade, non-pathogenic *St. thermophilus*-like microorganism with high potential as adjunct starter and bioprotective culture in cheese making (De Vuyst and Tsakalidou 2008) but has also been identified once as causative agent in human infection (Herrero et al. 1996).

Food is considered to be the most active vector spreading drug resistance between man and animals (European Commission 2005). Inadequate antibiotic treatment and subclinical antibiotic pressure establish an antibiotic resistance gene pool (Levy 2001), facilitating gene transfer among bacteria of different genera (Perreten et al. 1997). The ready availability and unsupervised distribution of antibiotics in developing countries and consumption of milk containing antimicrobial residues (Bonfoh et al. 2003a) fuels the problematic within susceptible populations of both human and animals exposed (Levy and Marshall 2004).

The spread of antibiotic resistant bacteria belonging to the SESBC in ready-to-eat artisanal sour milks from Western and Eastern Africa was investigated in this study.

6.4 Materials and Methods

6.4.1 Sample collection

29 raw and sour milk samples from production sites or local markets were collected between July 2007 and June 2008 in Mali, Kenya and Somalia (Table 16). These milks were filled into 50 ml Falcon tubes (Bioswisstec AG, Schaffhausen, Switzerland), frozen and transported in ice filled boxes to the laboratory of Food Biotechnology at ETH Zurich within one day and stored at -20°C until analysis.

6.4.2 Microbial analysis

Bacterial counts of thawed samples were determined by serial dilutions (10^{-1} – 10^{-6}) performed in sterile peptone water [0.85% NaCl (w/v) and 0.1% peptone (w/v) (VWR-International, Dietlikon, Switzerland)]. 0.1-ml aliquots were plated on a modified *St. macedonicus* semi-differential medium (SM) (Pacini et al. 2006) and SM containing 10 µg/ml tetracycline (SM_{Tet}) (Sigma-Aldrich, Buchs, Switzerland) agar plates and incubated aerobically at 45°C for 48-72 h to calculate the tetracycline prevalence of SBSEC bacteria in these samples. SM medium was prepared according to Pacini et al. (2006) with following changes: 35 g/l Count Agar Sugar free was replaced with 15 g/l peptone from casein (Merck, Darmstadt, Germany) and 5 g/l sodium chloride; 0.5 g/l potassium ascorbate was replaced with 0.5 g/l ascorbic acid (Sigma); and 19 g/l glycerol phosphate with 19 g/l disodium-glycerol phosphate (Sigma). Milk pH (pH-meter 780, Metrohm AG, Herisau, Switzerland) was measured of the same samples.

Five to ten colonies were picked based on color, shape, colony size and agar staining from SM and SM_{Tet} agar plates, respectively. Isolates were pure streaked three times on the same medium and subsequently pre-typed by Gram-staining (Gregersen 1978), catalase activity testing (3% H_2O_2) and microscopic inspection. Pure cultures were stored at -80°C in M17 (Labo-Life Sàrl, Pully, Switzerland) broth supplemented with 50% (v/v) glycerol.

The type strains *St. bovis* DSM 20480^T, *St. infantarius* subsp. *infantarius* CCUG 43820^T, *St. infantarius* subsp. *coli* CCUG 43822^T, *St. alactolyticus* DSM 20782^T, *St. gallolyticus* subsp. *gallolyticus* DSM 16831^T, *St. gallolyticus* subsp. *macedonicus* DSM 15879^T, *St. thermophilus* DSM 20617^T and *Enterococcus faecalis* DSM 20478^T were used as controls.

Table 16 Bacterial counts, tetracycline resistance prevalence, designation of Gram-positive catalse-negative bacteria, pH and designation of the 29 samples analyzed in this study

Sample Nr.	village	Product type	Sample pH	Designation of bacterial isolates[a]						Bacterial counts [Log_{10}(cfu/ml)]		Tetracycline resistance prevalence [%]
				SM			SM_{Tet}			SM	SM_{Tet}	
Mali				I	G	E	I	G	E			
28	Bankoumana	heated milk	5.3	n	3	n	6	n	n	8.3	5.8	0.3
29	Bankoumana	*Fènè*	3.8	n	n	2	n	n	n	< 2	< 1	n.d.
30	Bankoumana	*Lait Caillé*	3.8	n	n	5	n	n	n	4.8	< 1	n.d.
31	Koursalé	heated milk	6.6	n	n	2	1	n	3	4.1	3.9	96.5
32	Koursalé	*Fènè*	4.0	1	n	5	n	n	3	6.7	3.5	0.1
33	Koursalé	*Lait Caillé*	3.7	n	n	n	n	n	n	< 1	< 1	n.d.
34	Quelessebougou	heated milk	6.7	1	1	n	1	1	3	7.9	7.4	94.4
35	Quelessebougou	Raw milk	6.3	2	3	n	2	n	1	6.2	6.1	97.0
36	Kirina	heated milk	6.3	1	2	n	2	2	1	7.8	7.6	54.9
37	Kasela	Raw milk	6.6	n	n	1	2	n	1	7.8	6.5	6.1
38	Kasela	*Fènè*	4.2	n	2	n	4	n	n	7.7	3.3	<0.1
39	Bamako	*Fènè*	4.4	3	n	2	n	n	5	8.1	7.3	15.7
40	Djoliba	*Lait Caillé*	4.2	n	1	4	n	1	4	6.2	6.1	98.8
41	Kolé	heated milk	6.5	2	n	n	2	n	2	8.3	7.2	8.8
42	Djoliba	*Lait Caillé*	3.9	n	n	5	n	n	n	3.3	< 1	n.d.
43	Kéléya	*Fènè*	3.7	n	n	n	n	n	n	< 1	< 1	n.d.
44	Bamako	*Fènè*	4.0	n	n	n	n	n	n	< 1	< 1	n.d.
45	Bamako	*Fènè*	4.0	n	n	5	n	n	5	6.5	5.0	3.2
46	Samayana	*Lait Caillé*	4.0	1	n	4	1	n	4	6.8	5.8	10.6
	Somalia											
130	Garowe	*Suusac*	4.4	n	n	5	n	n	4	6.9	6.2	20.3
135	Garowe	*Suusac*	4.5	n	2	3	n	3	1	6.2	5.7	37.9
139	Garowe	*Suusac*	4.5	n	1	4	n	1	4	5.3	5.2	92.8
140	Garowe	*Suusac*	4.5	1	1	3	n	2	2	5.8	5.5	49.2
150	Burco	goat milk	5.1	3	n	3	4	n	1	6.6	5.7	13.0
	Kenya											
217	Manera	*Suusac*	4.1	3	n	1	n	n	10	6.3	6.0	47.2
218	Manera	*Suusac*	4.3	10	n	n	7	n	2	7.6	6.7	10.6
219	Manera	*Suusac*	4.4	7	n	3	9	n	1	6.9	6.7	97.1
220	Manera	*Suusac*	4.0	n	n	2	n	n	3	4.8	4.5	94.7
221	Manera	*Suusac*	4.4	10	n	n	9	n	n	8.3	8.0	45.8

n = not detected within the Gram-positive and catalase-negative colonies picked from SM and SM_{tet} agar plates.

[a] Number of Gram-positive an catalase-negative bacteria typed as *St. infantarius* subsp. *infantarius* (I), *St. gallolyticus* subsp. *macedonicus* (G) and enterococci (E).

6.4.3 Bacterial DNA extraction and molecular typing to strain level

Genomic DNA was extracted as previsously described (Goldenberger et al. 1995). PCR reactions were performed in a Biometra TGradient Cycler (BioLabo, Châtel-St. Denis, Switzerland). Isolates of the SBSEC were typed to species and subspecies level by PCR and RFLP (Chen et al. 2008) and to strain level by rep-PCR (Gevers et al. 2001) (Table 17). *St. gallolyticus* was typed to subspecies level by the *St. gallolyticus* subsp. *macedonicus* specific PCR (Papadelli et al. 2003) (Table 17).

Enterococci were identified at genus level by PCR (Ke et al. 1999) (Table 17) and thus separated from SBSEC bacteria. Agarose gel images from PCR-products, RFLP and rep-PCR (normalized to a 100-bp and 1-kb DNA ladder) banding patterns were captured with the AlphImager™ system (Alpha Innotech Corporation, San Leandro, California, USA) after staining in ethidium bromide (2.5 mg/l).

6.4.4 Phenotypic and genotypic antibiotic resistance profiling

The minimal inhibitory concentrations (MIC) of selected SBSEC bacteria were determined by microdilution (Klare et al. 2005) using *St. thermophilus* sensitive medium (SSM) (Tosi et al. 2007) with the following antibiotics: ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, neomycin, penicillin, streptomycin, tetracyline, trimethoprim and vancomycin. One isolate per rep-PCR cluster or when rep-PCR clusters were too similar, at least one isolated per sample region was selected for antibiotic resistance profiling. Microplates were incubated aerobically at 37℃ for 21-22 h. Isolates were designated as resistant if MIC values were higher than recommended guidelines for *Streptococcus* subsp. other than *St. pneumoniae* (CLSI 2006b) or if MIC values were clear outliers to strains of the same subspecies and at least two log steps higher than the MIC of the type strain. MIC values of the type strains were analyzed twice.

Antibiotc resitance genes were detected by microarray hybridization assay (Perreten et al. 2005) and ambiguous genes confirmed by PCR as described in Table 17.

Table 17 Oligonucleotide-primers and PCR conditions used in this study

Specifity	Primer	Primer sequence 5'→3'	PCR conitions	$MgCl_2$ [mM][a]	Reference
St. bovis /St. equinus complex	ES5-29F EL1265R[e]	TAAAACCHTTAGGHGAHCGWRTBGT CAAGTTCAAGTTCAGCAACTTTTG	95℃ 5 min [b]; 40 x (94℃ 30 sec, 58℃ 30 sec, 72℃ 2 min) [c]; 72℃ 10 min [d]	1.5	Chen et al., (2008)
St. gallolyticus subsp. *macedonicus*	16MAC BSR534/18	TAGTGTTTAACACATGTTAGAGA ATTACCGCGGCTGCTGGC	94℃ 2 min; 40 x (94℃ 30 sec, 57℃ 30 sec, 72℃ 30 sec); 72℃ 10 min	1.5	Papadelli et al., (2003)
Enterococcus	Ent1 Ent2	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	95℃ 5 min; 40 x (95℃ 30 sec, 55℃ 30 sec, 72℃ 60 sec); 72℃ 7 min	3	Ke et al., (1999)
rep-PCR	$(GTG)_5$	GTGGTGGTGGTGGTG	95℃ 5 min; 35 x (40℃ 60 sec, 65℃ 8 min, 65℃ 8 min); 65℃ 8 min	1.5	Gevers et al., (2001)
tet(S)	TetS-1 TetS-2	ATCAAGATATTAAGGAC TTCTCTATGTGGTAATC	95℃ 3 min; 35 x (95℃ 30 sec, 54℃ 30sec, 72℃ 60 sec); 72℃ 7 min	1.5	Leisibach, (2004)

[a] Each reaction mix contained 1µl extracted DNA, 1 µM of each primer (Microsynth), 0.2 mM dNTP (Amersham Biosciences), 2.5 U *Taq*-Polymerase (Euroclone, Italy), 2% Tween 20, 1x Reaction PCR buffer (Euroclone) an x mM $MgCl_2$ (Euroclone). The rep-PCR reaction mix contained additionally 4% DSMO. [b] Initial denaturation conditions. [c] Cycles x (denaturation, annealing, polymerization), [d] Final polymerization conditions. [e] The ratio of forward ES5-29F to reverse primer EL1265R was set to 5:1.

6.5 Results

6.5.1 Bacterial counts and molecular typing of isolates

Bacterial counts of Gram-positive and catalase-negative isolates were higher on SM than on SM_{tet} agar plates and between 3. 3 and 8.3 Log_{10}(cfu/ml) for 23 samples (Table 16). No colonies were found on SM_{tet} agar from sample 42. Bacterial counts on SM and SM_{tet} agar plates were below the detection limit of 10 cfu/ml for the samples 29, 30, 33, 43 and 44.

240 SBSEC bacteria and enterococci were picked from SM (n=120) and SM_{Tet} (n=120) plates. 95 isolates (45 SM / 50 SM_{Tet}) from 17 samples were typed as *St. infantarius* subsp. *infatarius* (55 Kenya, 31 Mali, 8 Somalia) and 26 (16 SM / 10 SM_{Tet}) from nine samples as *St. gallolyticus* subsp. *macedonicus* (16 Mali, 10 Somalia) (Table 16 Seven *St. gallolyticus* subsp. *macedonicus* strains tested negative for SBSEC bacteria displaying no color change around the colony on SM agar plates. The 26 *St. gallolyticus* subsp. *macedonicus* isolates were grouped into 9 strain clusters by rep-PCR (data not shown), of which one cluster included the type strain. Rep-PCR banding patters were too similar to divide *St. infantarius* subsp. *infantarius* into distinct strain clusters, but isolates from Malian samples were clearly distinguishable from those from Kenya and Somalia.

119 isolates (59 SM / 60 SM_{Tet}) from 22 samples were typed as enterococci (23 Kenya, 67 Mali, 29 Somalia) (Table 16) of which 33 tested positive for SBSEC bacteria by yellowing the SM agar around the colony.

6.5.2 Antibiotic resistance prevalence and profiling

Maximal tetracycline resistance prevalence was found in sample 40 with 98.8% (Table 1) and a median of the 29 samples of 11.8% both based on the ratio of SM_{tet} to SM counts.

13 *St. infantarius* subsp. *infantarius* from ten different samples and seven *St. gallolyticus* subsp. *macedonicus* from seven different rep-PCR clusters were selected for phenotypic antibiotic resistance profiling. Two multidrug-resistant, seven single-tetracycline resistant and four susceptible *St. infantarius* subsp. *infantarius* strains were detected, harboring the tetracycline resistance genes *tet*(L), *tet*(M) and *tet*(S) (Table 18, Figure 10).

Five *St. gallolyticus* subsp. *macedonicus* were resistant against tetracycline harboring the *tet*(M) gene (Table 18) and two were concomitant resistant against erythromycin and tetracycline harboring the *erm*(B) the *tet*(M) genes (Table 18, Figure 10). Strain T36P

harbored the erythromycin resistance gene *erm*(Q) and the tetracycline resistance gene *tet*(M) but displayed only tetracycline resistance (Table 18, Figure 10). Neomycin resistance could not be related to an acquired resistance gene included on the microchip used for genotypic antibiotic resistance profiling (Table 18).

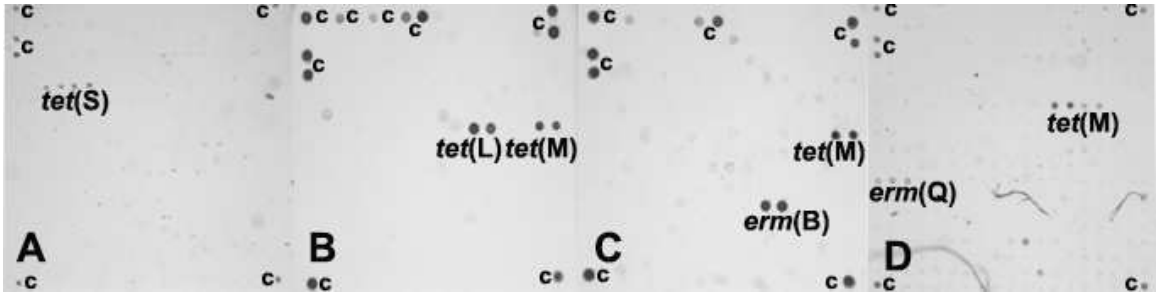

Figure 10 Micrograph of microarray hybridized with genomic DNA of *Streptococcus infantarius* subsp. *infantarius* T37T (A), *St. infantarius* subsp. *infantarius* T219I (B), *St. gallolyticus* subsp. *macedonicus* T40Q (C) and *St. gallolyticus* subsp. *macedonicus* T36P (D) isolated from *fènè* and suusac, respectively.

Genes encode for the following resistances: *tet*(L), *tet*(M) and *tet*(S) for tetracycline and *erm*(B) and *erm*(Q) for erythromycin. (c) biotin position markers.

Table 18 Phenotypic and genotypic antibiotic resistance profiles of selected isolates of *St. infantarius* subsp*. infantarius* and *St. gallolyticus* subsp. *macedonicus*

Strain[a]	**Phenotypic antibiotic resistance profile (MIC in [µg/mL])**[b]	**Resistance genes detected by microarray**[c]
St. infantarius subsp.		
infantarius CCUG 43820^T	none	none
T37T	TE(16)	*tet*(S)
39G	AM(0.5), C(256), E(4), GM(16), N(32), P(1),	none
T41P	TE(32)	*tet*(S)
150A	AM(0.5), C(128), CC(2), E(4), GM(16), P(0.5), S(>256), TE(128)	*tet*(L), *tet*(M)
T150B	TE(16)	*tet*(S)
T150D	TE(16)	*tet*(S)
T219I	TE(32)	*tet*(L), *tet*(M)
221E	TE(32)	*tet*(L), *tet*(M)
T221I	TE(32)	*tet*(L), *tet*(M)
St. gallolyticus subsp.		
macedonicus DSM 15879 ^{T}	none	none
28G	N(8)	none
T36S	E(>32), TE(32)	*erm*(B), *tet*(M)
T36P	TE(32)	*tet*(M), *erm*(Q),
T40Q	E(>32), TE(64)	*erm*(B), *tet*(M)
139C	N(8), TE(16)	*tet*(M)
T139D	TE(16)	*tet*(M)

[a] Strains with running numbers 28-46, 130-150 an 217-221 were isolated from samples from Mali, Somalia an Kenya, respectively (Table 16).

[b] AM: ampicillin, C: chloramphenicol, CC: clinamycin, E: erythromycin, GM: gentamicin, N: neomycin, P: penicillin, S: streptomycin, TE: tetracycline. T indicates the isolation from SM_{Tet} , letters behind numbers designate the individual isolates from the corresponding sample.

6.6 Discussion

Bacteria belonging to the SBSEC isolated from East and West African sour milks, which are important protein staples of these populations were for the first time unequivocally typed by semi-selective isolation, PCR, RFLP and rep-PCR (Chen *et al.*, 2008; Gevers *et al.*, 2001; Pacini *et al.*, 2006; Papadelli *et al.*, 2003) and profiled on antibiotic resistances. These bacteria are distinguishable from bacteria growing coexistent on SM agar plates due to yellowing of the agar (Pacini *et al.*, 2006). Low-rate raffinose acidifying *St. gallolyticus* subsp. *macedonicus* not yellowing the SM agar were reported in this study and by Pacini *et al.* (2006). These authors however reported no enterococcal strains yellowing the SM-media. Similar rep-PCR banding patterns for *St. infantarius* subsp. *infantarius* isolates originating from the same geographical area (Mali or Kenya/Somalia) are concordantly reported in this study and by Abdelgadir *et al.* (2008) (Sudan) and therefore PFGE might be more appropriate than rep-PCR for unequivocal strain discrimination, whereas *St. gallolyticus* subsp. *macedonicus* isolates were clearly classified by rep-PCR.

Elevated tetracycline resistance prevalence of SBSEC-bacteria from this study is related to uncontrolled antibiotic use of oxytetracycline and tetracycline as well as no adherence to recommended withdrawal times (Bonfoh *et al.*, 2003; Younan and Bornstein, 2007). Subclinical oxytetracycline pressure selects for tetracycline resistant bacteria leading to the formation of a tetracycline resistance gene pool among different LAB present in spontaneously fermented African sour milks (chapter 3 and 4; Jans unpublished data).

Clinical isolates of SBSEC-bacteria exhibited tetracycline, clindamycin and erythromycin resistance (Beck *et al.*, 2008; Corredoira *et al.*, 2008a). No breakpoints have been published for gentamycin or penicillin (CLSI, 2006; European community, 2005), which might be of interest due to *St. bovis* endocartits is treated with the combination penicillin G / gentamycin (David *et al.*, 2008).

6.7 Conclusion

There is no straight forward explanation why high cell numbers of *St. infantarius* subsp. *infantarius* and *St. gallolyticus* subsp. *macedonicus* were found in artisanal sour milks from Kenya, Somalia and Mali. Further research work has to elucidate if these species might be used as novel fermentative species like proposed for *St. gallolyticus* subsp. *macedonicus* or if they indicat a serious health hazard to human as found in human

medicin for *St. infanterius* subsp. *infantarius.* In this regard we found among both species a remarkably high tetracycline resistance gene pool and multi-drug resistant strains.

6.8 Acknowledgement

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7 *Vagococcus teuberi* sp. nov., isolated from artisanal sour milk *(fènè)* in Mali

Data presented in this chapter are submitted for publication to an international peer-reviewed journal in 2009 in a shorter version with the authorship:
Stephan Wullschleger, Clelia Seifert, Sarah Baumgartner, Christophe Lacroix, Bassirou Bonfoh and Leo Meile

16S rRNA gene sequence data are available in the DDBJ/EMBL/GenBank database under the accession numbers EU489723, FJ526386, FJ526387, FJ526388 and FJ526389 for strains DSM 21459T, GG-1, 9H, 24CA and CM-21, respectively.

7.1 Abstract

A Gram-positive, catalase-negative, coccoid bacterium (strain DSM 21459^T) was isolated at >10^8 colony forming units/ml from the Malian sour milk, *fènè* produced by spontaneous fermentation of cow milk. *Fènè* sour milk contain a consortium of lactic acid bacteria belonging to the genera *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Weissella*, *Lactococcus* and *Pediococcus*. The pure culture of strain DSM 21459^T is only weakly degrading lactose when grown in milk. Strain DSM 21459^T belongs to the genus *Vagococcus*, its DNA has a G+C content of 33.9 mol% and its 16S rRNA gene identity to the closest relatives *V. carniphilus* DSM 17031^T is 97.6% and to *V. fluvialis* DSM 5731^T 93.6% (*V. fluvialis* CCUG 32704 97.3%). DNA-DNA hybridizations between the genome of the new isolate and that of these type strains showed only a weak homology of 35.1% (*V. carniphilus*) and 16.5% (*V. fluvialis*), respectively, which supports a distinct species status of strain DSM 21459^T. This finding was also substantiated by discriminatory physiological and phenotypical characteristics such as acid production from saccharose and lack of acid production from mannitol in contrast to *V. fluvialis* and *V. carniphilus*, respectively. A 16S rRNA gene-based PCR approach was developed and validated as a specific assay targeting strains of this new species. Ten isolates from three independent fermentation processes were PCR-positive among 217 unknown cocci from 18 different *fènè* fermentations in this assay. They were further grouped according to their repetitive element PCR fingerprinting (rep-PCR) patterns into four different strain clusters.
Strain DSM 21459^T is therefore considered to belong to a novel species, for which the nomeclature *Vagococcus teuberi* sp. nov. is now proposed. The type strain is named DSM 21459^T (LMG 24695^T).

7.2 Key words

Vagococcus teuberi sp. nov., vagococci, lactic acid bacteria, spontaneous fermentation, sour milk, *fènè*

7.3 Introduction

Vagococcus fluvialis was the first species described in the genus *Vagococcus* that incorporated a phylogenetically distinct group of lactococci reacting with Lancefield group N antisera (Collins et al. 1989). *V. fluvialis* was originally isolated from chicken faeces and river water (Hashimoto et al. 1974) and typed as a motile *Lactococcus lactis*-like strain with an unresolved phylogenetic position (Schleifer et al. 1985). *V. fluvialis* was later associated with human or animal infections in three studies (Al-Ahmad et al. 2008; Pot et al. 1994; Teixeira et al. 1997). Five species of the genus *Vagococcus* have subsequently been described: *V. salmoninarum* isolated from rainbow trout (Wallbanks et al. 1990) recently shown to cause severe infections in its host (Ruiz-Zarzuela et al. 2005; Schmidtke and Carson 1994), *V. lutrae* and *V. fessus* isolated from other aquatic animals (Hoyles et al. 2000; Lawson et al. 1999) and *V. carniphilus* discovered in ground beef (Shewmaker et al. 2004) and dry sausages (Ammor et al. 2005) containing *V. carniphilus* strains displaying antimicrobial activity (Ammor et al. 2006) The last isolated member of genus *Vagococcus* to be typed to the species *V. elongatus* was isolated from a swine-manure storage pit (Lawson et al. 2007)

In this report we describe a polyphasic taxonomic study with one out of four identified strains (10 isolates), which were isolated from different spontaneously fermented milk *(fènè)* stocks at home- and small-scale production sites in Mali (chapter 2). Briefly, raw or heated cow milks were subjected to spontaneous overnight (22 h) fermentation at ambient temperatures (30.6 ± 5.3℃) for domestic or small-scale *fènè* production. On the basis of our results we consider that these four strains belong to an as yet undefined novel species of genus *Vagococcus*.

7.4 Material and methods

7.4.1 Microorganisms and culture conditions

An unknown bacterial isolate was picked as a white, round colony (1 mm in diameter) from a KF *Streptococcus* agar plate (Labo-Life Sàrl, Pully, Switzerland) at the 10^{-8}-dilution of a *fènè* sample obtained from household production in Kasséla, a village near Bamako, the capital city of Mali (chapter 2). This isolate was later deposited at the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) under the number DSM 21459^{T}. The strain was isolated from the final product *(fènè)*

after 22 h of fermentation, whereas GG-1, another isolate used for comparative DNA-DNA hybridization analysis was retrieved from the 10^{-1}-dilution of a sample taken after 4 h from the same spontaneous fermentation process. *Vagococcus carniphilus* DSM 17031^T (Shewmaker et al. 2004) was included as a control in several experiments. Both *V. carniphilus* DSM 17031^T and strain DSM 21459^T were routinely cultured at 37°C in TSYE medium containing 30 g/l tryptic soy broth (Becton Dickinson, Allschwil, Switzerland) and 3 g/l yeast extract (VWR International, Dietikon, Switzerland), under aerobic conditions. Growth behavior of strain DSM 21459^T was studied at 30°C in UHT milk with a 3.5%-milk fat content and compared to growth in MRS-lactose (Labo-Life Sàrl) containing 20 g/l lactose at 30°C.

7.4.2 Phenotypic and physiological characteristics of strain DSM 21459^T

Cell morphology of DSM 21459^T was analyzed by light microscopy (DM 1000, Leica, Wetzlar, Germany) and electron microscopy (Gemini 1530, Zeiss, Oberkochen, Germany). Gram-staining was carried out with KOH lysis (Gregersen 1978), catalase activity tested with 3% H_2O_2 and cell motility examined by light microscopy. Growth parameters, e.g. growth temperatures and aeration, pH and salt tolerance (4% and 6.5% NaCl), as well as other phenotypic assays were carried out in TSYE medium. Gas production was measured in MRS-glucose broth (Labo-Life Sàrl). Haemolytic activity of strain DSM 21459^T was tested by culturing it on Columbia agar plates with 5% sheep blood. Tellurite tolerance on TSYE agar plates was recorded with a 0.04% tellurite concentration and serological reactions monitored with the Slidex Strepto-Kit (bioMérieux, Geneva, Switzerland). Enzyme activity and acid production from different carbon sources were both tested for all species of the genus *Vagococcus* including four representative strains of the new species (DSM 21459^T as type strain) with the API Rapid ID with the API Rapid ID 32S system (bioMérieux). Enzyme activity and acid production from different carbon sources were both tested concurrently for all species of the genus *Vagococcus* and further detected DSM 21459^T strains with the API Rapid ID 32S system (bioMérieux). Lactose, glucose and galactose consumption as well as lactate, acetate and formate formation during bacterial growth assays were measured by HPLC (Doleyres et al. 2005). Strain DSM 21459^T was spot-inoculated on CASO agar plates (VWR-International) containing 5% skim milk and incubated aerobically at 37°C for 15 hours to test for proteolytic activity by precipitation of unhydrolyzed casein (Lindquist 1975). *Bacillus subtilis* subsp. *subtilis* DSM 4424 was used as positive control

for proteolytic activity. A phenotypic antibiotic resistance profile of strain DSM 21459^T was determined with 24 different antibiotics by disc diffusion tests on LSM-agar plates and with 18 antibiotics by micro-dilution tests on LSM-broth to obtain minimal inhibitory concentration (MIC) values (Klare et al. 2005) (Table 19). Fatty acid composition of bacterial cell walls was measured by DSMZ according to published methods (Kämpfer and Kroppenstedt 1996; Kuykendall et al. 1988; Miller 1982).

7.4.3 Genotypic and phylogenetic analysis

Total DNA was extracted according to Goldenberger et al. (1995) and 16S rRNA gene sequence of strain DSM 21459^T amplified using the bak4 (Greisen et al. 1994) and bak11w (Goldenberger et al. 1997) primers in subsequent sequencing of the 16S rRNA gene (Microsynth, Balgach, Switzerland) [accession nr. EU489723].

The *tuf* gene sequence of strain DSM 21459^T was amplified with primers U1 and U2 (Ke et al. 1999) and sequenced [accession Nr. FJ169507]. Comparative nucleotide analyses for identification of close relatives were executed through BLAST databank search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and FASTA analysis (http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi_rm=select). Whole genome DNA-DNA hybridization of strain DSM 21459^T with *V. carniphilus* DSM 17031^T, *V. fluvials* DSM 5731^T and isolate GG-1 was performed at DSMZ according to established protocols (Cashion et al. 1977; Huss et al. 1983; Ley et al. 1970). DSMZ also determined the genomic G+C content of strain DSM 21459^T by HPLC (Mesbah et al. 1989). Strain DSM 21459^T was investigated for the presence of plasmids using a small-scale plasmid isolation procedure (Anderson and Mc Kay 1983). Antibiotic resistance gene profiling of DSM 21459^T was assayed using a microarray hybridization protocol (Perreten et al. 2005). *Streptococcus pseudintermedius* KM1381 (Descloux et al. 2008) was used as positive control to confirm AR genes by PCR. PCR primers were designed on the *dfr(G)* gene sequence (accession number FM204877). A phylogenetic tree based on 16S rRNA gene sequences was constructed with the neighbour-joining method using *MEGA* version 4 (Tamura et al. 2007) and its robustness was validated with bootstrap analysis after 1000 replicates.

7.4.4 Molecular typing of the novel *Vagococcus* species

We developed a novel PCR approach which is capable to assign bacterial isolates into the new *Vagococcus* species represented by strain DSM 21459^T. It was designed *in*

silico through comparison of 16S rRNA gene sequence with homologous sequences of the six known *Vagococcus* species, 17 *Enterococcus* species and *Carnobacterium mobile* DSM 4848^T retrieved from the GenBank (Table 20) with the FASTA and Muscle Multiple Alignment (http://www.bioinformatics.nl/tools/muscle.html) programs. The novel primer pair and PCR conditions were validated experimentally with 38 species from eight different genera (Table 20). Finally, the novel species-specific PCR was applied on 217 as yet untyped isolates from spontaneous *fènè* fermentation processes (chapter 2). Bacterial isolates testing positive in this novel species-specific PCR assay were further differentiated with repetitive element PCR fingerprinting (rep-PCR) using the $(GTG)_5$ primer as described elsewhere [10]. rep-PCR banding patterns were normalized to a TriDye 100-bp and 1-kb DNA ladder (BioConcept, Allschwil, Switzerland) and visually inspected.

Table 19 Microdilution (MIC[§] values) and disc diffusion (DIZ° values) assays de termining the phenotypic antibiotic resistance profile of strain DSM 21459^T

Antibiotic[1]	**AM**	**AMC**	**AMO**	**C**	**CC**	**CIP**	**CLO**	**E**	**FA**
MIC[§]	0.25	n.t.	≤0.125	4	0.5	n.t	2	8	≤0.016
DIZ°	13	0	n.t.	20	10	12	n.t.	10	22
Antibiotic	**GM**	**K**	**MUP**	**N**	**NA**	**NV**	**OFX**	**OX**	**P**
MIC[§]	4	31.5	16	n.t	128	8	n.t.	n.t.	0.125
DIZ°	12	12	15	10	n.t.	0	0	0	19
Antibiotic	**QD**	**RA**	**STR**	**SXT**	**T**	**TE**	**TRI**	**VA**	
MIC[§]	n.t.	≤0.125	2	n.t.	n.t.	64	256	≤0.125	
DIZ°	20	17	0	0	0	0	n.t	13	

[§]; minimal inhibitory concentration [µg/ml], °; diameter of the inhibition zone, disc diameter is 6 mm, n.t.; not tested, [1] Antibiotic content of discs in µg.

AM: ampicillin (antibiotic concentration of the disc, 10 µg); AMC: amoxicillin/clavulanic acid (20/10 µg) ; AMO: amoxicillin; C: chloramphenicol (30 µg); CC: clindamycin (2 µg); CIP: ciprofloxacin (5 µg) ; CLO: cloxacillin; E: erythromycin (15 µg); FA: fusidic acid (10 µg); GM: gentamycin (10 µg); K: kanamycin (30 µg); MUP: mupirocin (200 µg); N: neomycin (30 µg); NA: nalidixic acid; NV: novobiocin (5 µg); OFX: ofloxacin (5 µg); OX: oxacillin (1 µg); P: penicillin G (10 units); QD: quinopristin/dalfopristin (15 µg); RA: rifampicin (30 µg); STR: streptomycin (25 µg); SXT: trimethoprim/sulfamethoxazol (1.25/23.72 µg); T: oxytetracycline (30 µg); TE: tetracycline (30 µg); TRI: trimethoprim; VA: vancomycin (30 µg).

7.4.5 Statistical analysis

Bacterial counts were normalized by Log_{10} transformation. Differences between means were tested for statistical significance (p=0.05) using the ANOVA Tukey's-b test or the independent t-test of SPSS software (ver. 16.0 SPSS, Chicago, IL, USA).

Table 20 16S rRNA gene sequences of bacterial strains used in species-specific PCR typing of the novel *Vagococcus* species and experimental validation

Strains	Culture collection accession number	GenBank accession number of 16S r DNA sequences
*C. mobile**,°	DSM 4848[T]	AB083414
*E. asini**	DSM 11492[T]	Y11621
*E. casseliflavus**,°	DSM 20680[T]	AJ420804
*E. devriesie**	LMG 14595	AJ891167
*E. durans**,°	DSM 20633[T]	AJ420801
*E. faecalis**,°	DSM 20478[T]	AJ420803
*E. faecium**,°	DSM 20477[T]	AJ276355
*E. gallinarum**,°	DSM 20628[T]	AJ420805
*E. hirae**,°	DSM 20160[T]	AJ420799
*E. malodoratus**	DSM 20681[T]	AF061012
*E. pseudoavium**	DSM 5632[T]	DQ411809
*E. raffinosus**	DSM 5633[T]	Y18296
*E. rotate**	CCM[d] 4629	AJ276352
*E. saccharominimus**	LMG 21727[T]	AJ626902
*E. silesiacus**	LMG 23085[T]	AM039966
*E. sulfureus**,°	DSM 6905[T]	DQ411815
*E. termitis**	LMG[c] 8895[T]	AM039968
*E. villorum**	DSM 15688	AF335596
Lc. lactis subsp. *cremoris*°	DSM 20069[T]	n.a.
Lc. lactis subsp. *lactis*°	DSM 20481[T]	n.a.
Lc. lactis subsp. *diacetylactis*°	UL719/RLB 22[e]	n.a.
Lc. garvieae°	DSM 20684[T]	n.a.
Lc. plantarum°	DSM 20686[T]	n.a.
Lb. plantarum°	DSM 20205	n.a.
P. acidilactici°	DSM 20284[T]	n.a.
P. pentosaceus°	DSM 20336[T]	n.a.
St. bovis°	DSM 20480[T]	n.a.

St. thermophilus°	DSM 20617^T	n.a.
*V. carniphilus**,°	DSM[a] 17031^T	AY179329
*V. elongatus**,°	CCUG[b] 51432 ^{T}	AF445297
*V. fessus**,°	DSM 15697^T	AJ243326
*V. fluvialis**,°	DSM 5731^T	X54258
*V. lutrae**,°	DSM 15731^T	Y17152
*V. salmoninarum**,°	DSM 6633^T	X54272
W. confusa°	DSM 20196^T	n.a.
W. paramesenteroides°	DSM 20288^T	n.a.

Abbreviations of genera: *C. = Carnobacterium; E. = Enterococcus; Lc. = Lactococcus; Lb. = Lactobacillus*; *P. = Pediococcus; St. = Streptococcus; V. = Vagococcus; W. = Weisella.*

[a] German Collection of Microorganism and Cell Cultures (DSMZ), Braunschweig, Germany.

[b] Culture Collection University of Goteborg (CCUG), Goteborg, Sweden.

[c] Laboratory for Microbiology, University of Ghent (BCCM/LMG), Ghent, Belgium.

[d] Czech Collection of Microorganisms, Masaryk University, Brno (CCM), Brno, Czech Republic.

[e] Culture Collection of the Laboratory of Food Biotechnology, ETH Zurich, Switzerland.

n.a. = not applied, * Strains used for designing the primers, ° Strains used for validating the PCR.

7.5 Results

7.5.1 Phenotypic and physiological characteristics

Strain DSM 21459^T is a Gram-positive, catalase-negative, non-motile diploid coccus with pili-like extensions on the cell surface (Figure 11A), elongated in the direction of the chain and may occur in pairs or short chains (Figure 11B). Strain DSM 21459^T grows in a temperature range between 10℃ - 41℃ within 24 h in TSYE medium. Highest and similar growth rates were found between 30℃ and 37 ℃ at a μ_{max} of 0.92/h. Bacterial counts increased from 6.5±0.1 Log_{10}(cfu/ml) to 8.5±0.1 Log_{10}(cfu/ml) at 30℃ and 37℃ within 6 h. Strain DSM 21459^T grows in TSYE containing 6.5% NaCl, at pH 4.7 and pH 9.0, but not at pH 9.6. It is a facultative anaerobe, and does not produce gas when grown in MRS-glucose broth. The strain is γ-haemolytic and does not tolerate levels of 0.04% tellurite. Serogrouping tests negative for the antigens A, B, C, D, F and G. Strain DSM 21459^T produces *β*-glucosidase, pyroglutamic acid arylamidase and glycyl-tryptophan arylamidase, but not alanyl-phenylalanyl-proline arylamidase, *N*-acetyl-*β*-glucosaminidase, and *β*-mannosidase, respectively. It produces acid from methyl-*β*-D-glucopyranoside but not from arabitol, cyclodextrin and pullulane. The predominant cell envelope fatty acids are oleic (33.6%), palmitic (28.2%) and stearic (17.2%) acids. Other

phenotypic differences from *Vagococcus* species are shown in Table 21. Since no proteolytic activity was detected in strain DSM 21459^T, it was cultered in milk to test lactose as the major carbon and energy source. Indeed, only a slight but significant growth of the strain was observed in milk at 30℃ a fter 24 h. Bacterial counts increased from 7.0±0.1 Log_{10}(cfu/ml) to 8.1±0.1 Log_{10}(cfu/ml) as pH decreased from 6.6±0.0 to 6.4±0.1. Strain DSM 21459^T initially synthesized 0.1 g/l acetate over 24 h time period,

Table 21 Physiological characteristics of *Vagococcus* species type strains and inter-species differences of species DSM 21459^T

All strains produced no acid from lactose, raffinose, arabinose, arabitol, glycogen, melibiose, melezitose, tagatose, were negative in the Voges-Proskauer test and did not hydrolyse hippurate. No strain produced *α*- or *β*-galactosidase, *β*-glucuronidase, alkaline phosphatase, ala-phenylalanine-proline-arylamidase, *β*-mannosidase or urease.

Name	*V. teuberi* DSM 21459^T	*V. teuberi* 9H	*V. teuberi* 21CM	*V. teuberi* 24CA	*V. carniphilus* DSM 17031^T	*V. fluvialis* DSM 5731^T	*V. lutrae* DSM 15741^T	*V. fessus* DSM 15697^T	*V. salmoninarum* DSM 6633^T	*V. elongates* DSM 51432^T
Enzymes:										
arginine dihydrolase	-	+	-	-	+[a]	-	-	+	-	-
β-glucosidase	+	+	+	+	-	+	+	-	+	-
N-acetyl-β-glucosaminidase	-	-	-	-	-	-	+	-	-	-
glycyl-tryptophan arylamidase	+	+	+	+	+	+	+	-	+	+
Acid production from:										
D-ribose	+	+	+	+	+	+	-[a]	-	+	-
D-mannitol	-	-	-	-	-	+	-	-	-	-
D-sorbitol	-	-	-	-	-	+	+	-	-	-
D-lactose	-	-	+	-	-	-	-	-	-	-
D-trehalose	+	+	+	+	+	+	+	-	+	-
D-sucrose	+	+	+	+	-	-	+	-	+	-
α-cyclodextrin	-	+	-	-	+	+	+	-	+	-
pullulan	-	-	-	-	-	+	-	-	-	-
D-maltose	+	+	+	+	+	+	+	-	+	-
methyl β-D-glucopyranoside	+	+	+	+	+	-	+	-	-	-
D-tagatose	-	-	-	+	-	-	-	-	-[a]	-

[a] different to literature(Lawson et al. 1999; Shewmaker et al. 2004; Wallbanks et al. 1990)

+, positive; -, negative.

and this level remained constant over an extended 96 h fermentation trial. Neither lactate nor formate were found (detection limit 0.02 g/l) during the 96 h trials of strain DSM 21459T incubated in milk even though 0.9 g/l lactose and 0.1 g/l galactose were metabolized during this period.

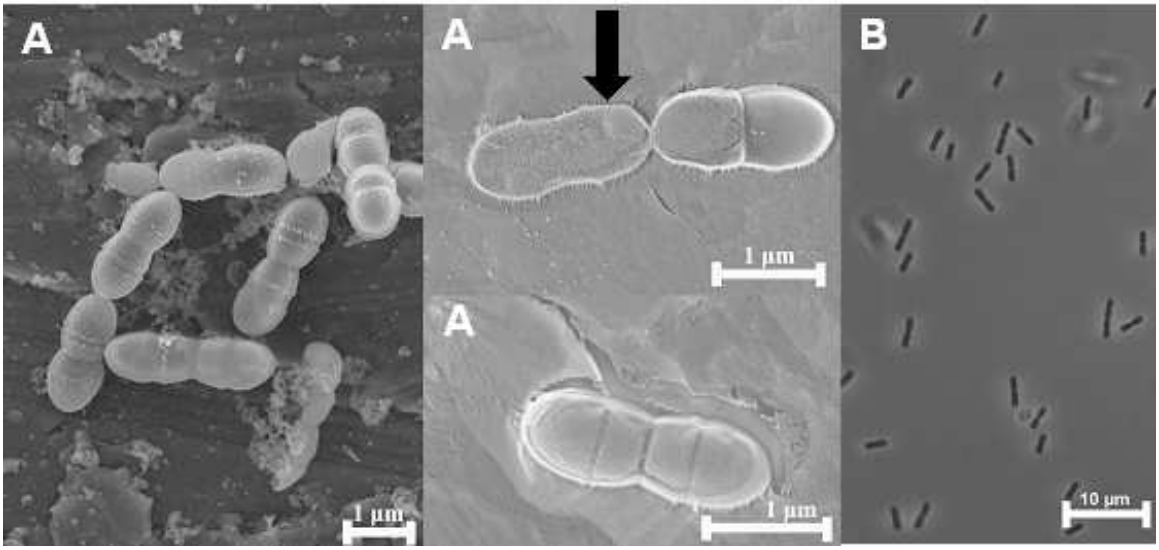

Figure 11 Electron micrographs [S. Handschin, Electron Microscopy ETH Zurich (EMEZ)] (A) indicating pili-like extensions on the cell surface (➞) and light micrograph (B) of DSM 21549^T.

Higher metabolic activities were detected in MRS-lactose and TSYE medium. Strain DSM 21459^T produced 0.4 g/l acetate, 0.3 g/l lactate and 0.5 g/l formate within 24 h at 30℃ and used 0.9 g/l lactose and 0.1 g/l galactose during 44 h in MRS-lactose. The strain metabolized 2.7 g/l glucose and 0.7 g/l galactose in TSYE within 24 h leading to formation of 1.4 g/l lactate, 0.6 g/l acetate and 0.8 g/l formate at 30℃. It is evident that milk is a sub-optimal growth medium for pure cultures of strain DSM 21459^T and lactose a poor carbon source. Milk lactose can therefore not be responsible for the high titer of >10^8 cfu/ml observed for strain DSM 21459^T in *fènè* sour milk containing other lactic acid bacteria.

Antibiotic resistance of strain DSM 21459^T was comprehensively profiled through disc diffusion and microdilution tests as proposed by Klare et al. (2007). Strain DSM 21459^T grew well in the newly developed LSM medium (Klare et al. 2005) for antibiotic susceptibility testing of non-*Enterococcus* lactic acid bacteria. No halo formation was detected around tetracycline, oxytetracycline, trimethoprim/sulfamethoxazol, amoxicillin/clavulanic acid, novobiocin, ofloxacin, oxacillin and streptomycin in disc

diffusion tests indicating phenotypic antibiotic resistance to these antibiotics (Table 19). High MIC-values at 64 µg/ml and 256 µg/ml were observed in microdilution assays for tetracycline and trimethoprim (Table 19) when strain DSM 21459^T was compared to susceptible *Enterococcus* strains.

7.5.2 Genotypic and phylogenetic analysis

Initial comparative analysis of a 1548-bp fragment of the 16S rRNA gene of the unknown strain DSM 21459^T with homologous sequences of other *Vagococcus* species revealed identity values of less than 98%. The highest homology was found with the 16S rRNA gene of *V. carniphilus* DSM 17031^T of 97.6% and with *V. fluvialis* DSM 5731^T of 93.6%. The relationship of the unknown bacterium to other species among the genus *Vagococcus* is represented in a phylogenetic tree using neighbour-joining analysis of 16S rRNA gene sequences (Figure 12). Whole genome DNA-DNA hybridization of strain DSM 21459^T to these two type strains resulted in a DNA homology of 35.1% and 16.5%, respectively, clearly substantiating that strain DSM 21459^T represents a new species (Wayne et al. 1987). DNA homology of strain DSM 21459^T with the strain GG-1 is 102.1%. The strains GG-1 and DSM 21459^T therefore belong to the same species and even to the same strain-cluster. This confirms the outgrowth of strain GG-1 from 10^1 to 10^8 cfu/ml during a spontaneous *fènè* fermentation process. The G+C content in the genome of strain DSM 21459^T is 33.9 mol%. Strain DSM 21459^T contains several plasmids, the smallest one has a size of 4.5 kb (data not shown). Strain DSM 21459^T harbours the trimethoprim resistance inducing gene *dfr*(G) (confirmed by PCR) encoding a S2-dihydrofolate reductase, a gene also found in *Staphylococcus heamolyticus* and *Listeria monocytogenes* (Coque et al. 1999). Trimethoprim resistance was the only genotypic resistance, which we detected through microarray hybridization, whereas no tetracycline resistance gene target was found in this assay Figure 13.

7.5.3 Molecular typing and genotypic and phenotypic inter-species differences of the novel *Vagococcus* species

Growth of strain DSM 21459^T in spontaneous *fènè* fermentation processes suggested the presence of different strains belonging to the same species. We therefore developed a species-specific PCR for typing isolates belonging to the same species as DSM 21459^T. We finally selected the primer pair bakw11_CS [5'-AGTTTGATCMTGGCTCAG GACG-3'] and va-r1 [5'-TGATTCCACCATGCGGTAAAATC-3'] that amplified a 207-bp

fragment of the 16S rRNA gene of strain DSM 21459^T at the annealing temperature of 63℃ in the PCR assay (Figure 14). This assay was v alidated with 38 species belonging to 8 genera (Table 19). Only *Weissella paramesenteroides* DSM 20288^T displayed a weak band at approximately 200 bp (Figure 14C line 3). Unspecific bands were recorded at approximately 300 bp for *Lactococcus lactis* subsp. *cremoris* (Figure 14C line 9) and *Lc. plantarum* DSM 20686^T (Figure 14C line 13) and at approximately 550 bp for *V. fluvialis* DSM 5731^T (Figure 14A line 3).

These bands do not detract from the specificity of the PCR assay. Ten out of 217 unknown isolates from spontaneous *fènè* fermentation processes showed positive banding with the novel species-specific PCR assay. These ten isolates were grouped with rep-PCR into the four strain clusters named A, B, C and D (Figure 15) containing the isolates DSM 21459^T (lane 1), CG-22 (lane 2), GG-1 (not shown) in cluster A, 9H (lane 3) in cluster B, CM-21 (lane 4) and CM-22, CM-11, CM-12 (all not shown) in cluster C and 24CA (lane 5) and 24CB (not shown) in cluster D. These banding patterns were completely different from those of the type strains of the six *Vagococcus* species described to date. The clustered strains DSM 21459^T, 24CA, 9H and CM-21 could be further discriminated from one another by differential plasmid profiling and 16S rRNA gene homology (data not shown). These four strains displayed similar phenotypic antibiotic resistance profiles with high tetracycline MIC values (16 – 128 µg/ml) with sole exception of strain DSM 21459^T when exhibiting a high trimethoprim MIC value at >256 µg/ml (Table 19) compared to the other three strains with 0.5 to 2 µg/ml. Phenotypic inter-species differences were observed for strain 9H displaying arginine dihydrolase activity and acid production from α-cyclodextrin, strain 24CA producing acid from D-tagatose and strain CM-21, which metabolises lactose. Strain CM-21 acidified milk to pH 5.8 when grown overnight (18 h) at 37℃.

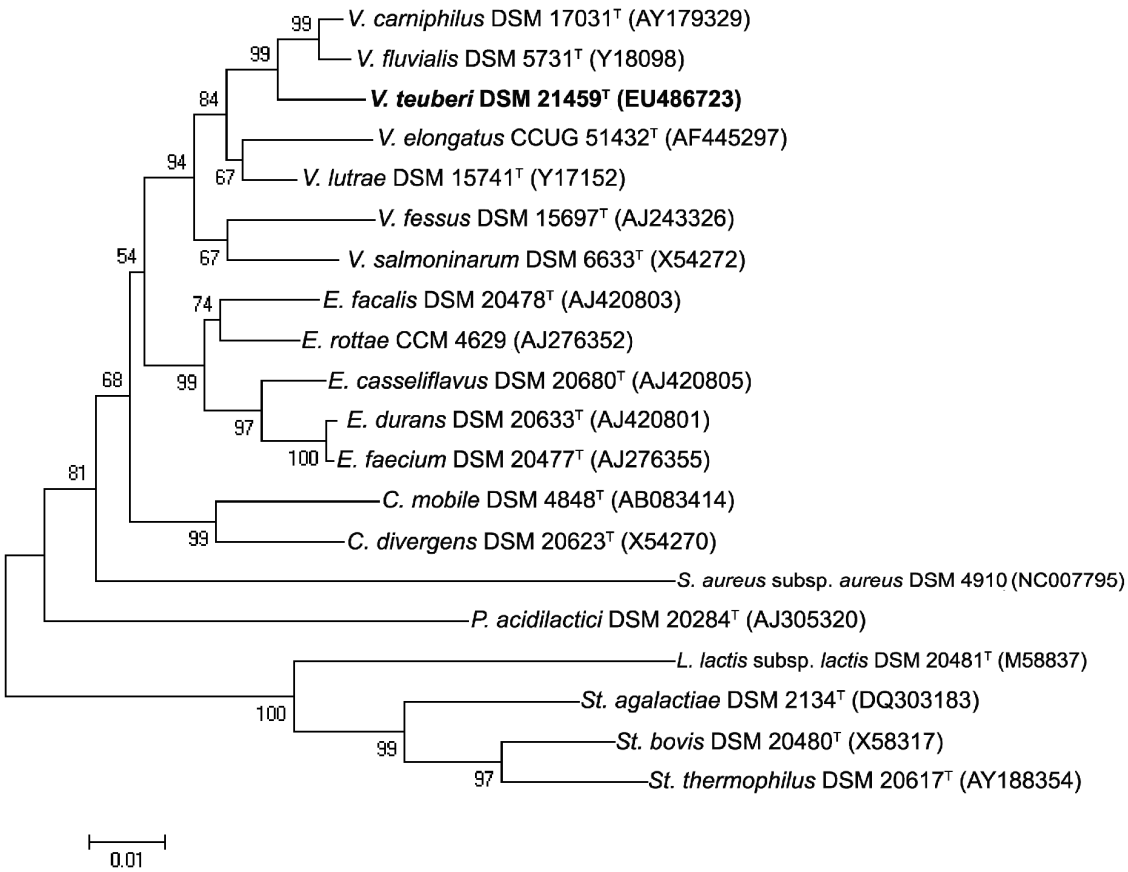

Figure 12 Phylogenetic tree showing the relationship of strain DSM 21549^T within other species of *Vagococcus* and other related genera.

The tree constructed in MEGA 4 using the neighbour-joining method (Tamura et al., 2007) was based on comparative assay of ca. 1230 nucleotides in 16S rRNA gene sequences. Bootstrap values at the nodes are displayed as percentages and were analysed with 1000 replicates. Bar = 0.01 substitutions per nucleotide position. Abbreviations of genera are indicated in Table 20. Numbers in parenthesis refer to 16S rRNA gene GenBank accession numbers.

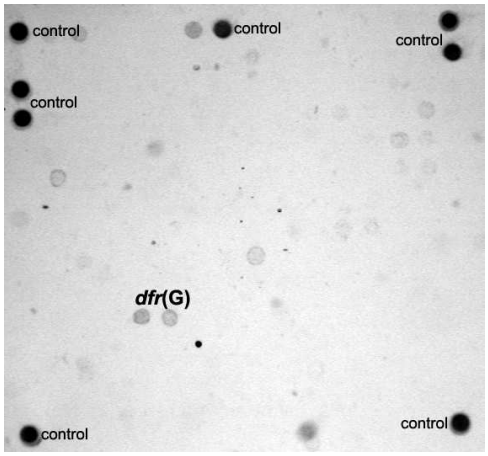

Figure 13 Microchip array hybridization with genomic DNA of strain DSM 21549^T to 90 antibiotic resistance genes, testing positive for the trimethoprim resistance inducing gene *dfr*(G).

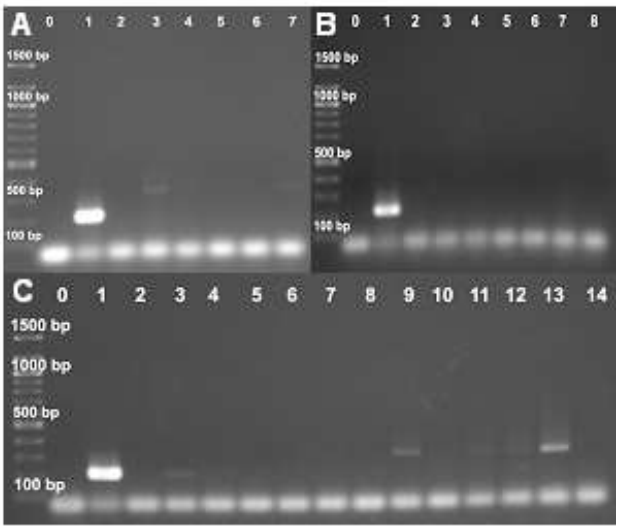

Figure 14 Gel-electrophoresis pattern of species-specific PCR amplicons based on primer pair bakw11_CS and va-r1 for typing isolates of the novel *Vagococcus* species.

Agarose gel A: Lane 0: negative control; 1: DSM 21549^T; 2: *V. carniphilus* DSM 17031^T; 3: *V. fluvialis* DSM 5731^T; 4: *V. lutrae* DSM 15741^T; 5: *V. fessus* DSM 15697^T; 6: *V. salmoninarum* DSM 6633^T; 7: *V. elongatus* CCUG 51432^T; Agarose gel B: Lane 0: negative control;1: DSM 21549^T; 2: *E. sulfureus* DSM 6905^T; 3: *E. gallinarum* DSM 20628^T; 4: *E. casseliflavus* DSM 20680^T; 5: *E. hirae* DSM 20160^T; 6: *E. malodoratus* DSM 20681^T; 7: *E. faecalis* DSM 20478^T; 8: *E. durans* DSM 20633^T; Agarose gel C: Lane 0: negative control; 1: DSM 21549^T; 2: *C. mobile* DSM 4848^T; 3: *W. paramesenteroides* DSM 20288^T; 4: *W. confusa* DSM 20196^T; 5: *P. pentosaceus* DSM 20336^T; 6: *P. acidilactici* DSM 20284^T; 7: *St. bovis* DSM 20480^T; 8: *St. thermophilus* DSM 20617^T; 9: *Lc. lactis* subsp. *cremoris* DSM 20069^T; 10: *Lc. lactis* subsp. *diacetylactis* UL719/RLB 22; 11: *Lc. lactis* subsp. *lactis* DSM 20481^T; 12: *Lc. garvieae* DSM 20684^T; 13: *Lc. plantarum* DSM 20686^T; 14: *Lb. plantarum* DSM 20205.

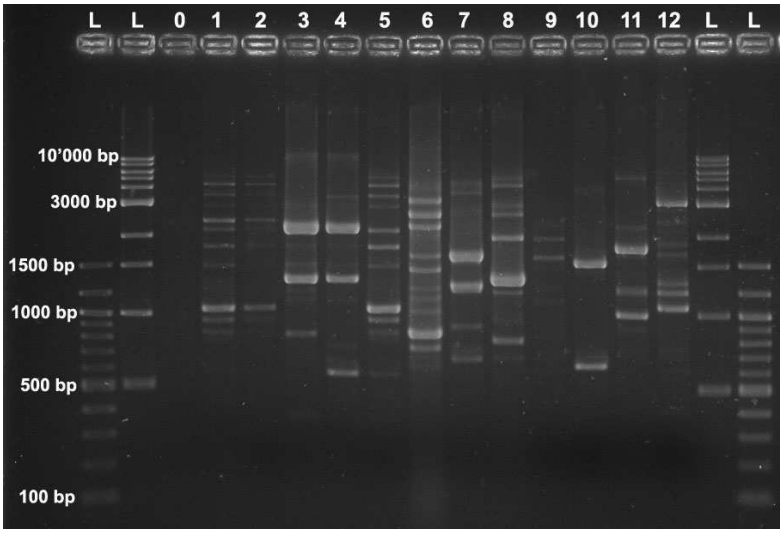

Figure 15 Agarose gel-electrophoresis with rep-PCR patterns from the four strain clusters A, B, C and D comprising the novel *Vagococcus* species and type strains of known *Vagococcus* species. Lane 0: negative control, including reaction buffer, $MgCl_2$, primers, nucleotides and Taq-polymerase, but lacking DNA template ; 1: cluster A / strain DSM 21549^T; 2: cluster A / isolate CG-22; 3: cluster B / isolate 9H; 4: cluster C / isolate CM-21; 5: cluster D / isolate 24CA; 6: isolate 10HS-111 not belonging to any vagococci; 7: *V. carniphilus* DSM 17031^T; 8: *V. fluvialis* DSM 5731^T; 9: *V. lutrae* DSM 15741^T; 10: *V. fessus* DSM 15697^T; 11: *V. salmoninarum* DSM 6633^T; 12: *V. elongatus* CCUG 51432^T; L: DNA size ladder.

7.6 Discussion

The undefined isolate, DSM 21459^T, was classified with polyphasic typing to the genus *Vagococcus* and further substantiated through physiological, phenotypic and genotypic assays. Strain DSM 21459^T could be clearly differentiated from other known *Vagococcus* species; according to 16S rRNA gene analysis the closest species in terms of phylogenetic relatedness are *V. carniphilus* and *V. fluvialis* (Figure 12). Strain DSM 21459^T could not be grouped in one of the currently described species since the genome homology of the new isolate measured by DNA-DNA hybridization with the type strains of both species was far below the discriminatory 70%-value (Wayne et al. 1987). Strain DSM 21459^T is therefore a novel species within the genus *Vagococcus*.

Vagococci are not commonly associated with food fermentation and milk represents a novel habitat for this genus. Slight but significant single-strain growth of strain DSM 21459^T in milk was observed. This is most likely due to an inherently low lactose metabolism and lack of proteolytic activity observed in DSM 21459^T when grown in milk. The fact that DSM 21459^T grew in a spontaneous *fènè* fermentation process from 10^1 to

10^8 cfu/ml, shown with a DNA-homology of 102.1% to the strain GG-1, precludes synergistic interaction with the complex *fènè* microbiota of streptococci, enterococci, lactobacilli, *Weissella* sp., lactococci and pediococci as seen in other artisanal sour milk and cheese (Teuber 1995).

Electron micrographs display pili-like extensions on the cell surface (Figure 11), previously unreported for other *Vagococcus* species (Collins et al. 1989; Hoyles et al. 2000; Lawson et al. 2007; Lawson et al. 1999; Shewmaker et al. 2004; Teixeira et al. 1997; Wallbanks et al. 1990).

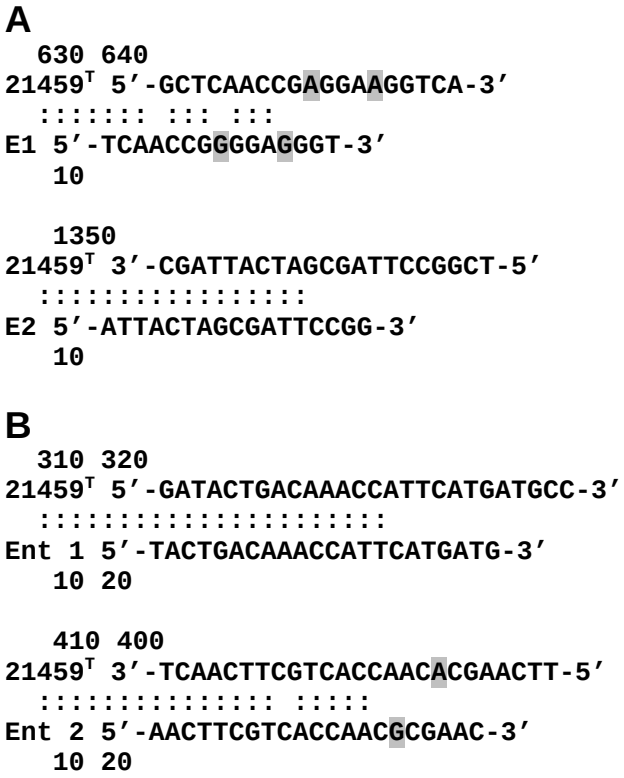

Figure 16 Comparison of the 16S rRNA gene sequence of DSM 21549^T with *Enterococcus*-specific primers E1 and E2 (Deasy et al., 2000) (A) and the tuf gene sequence of DSM 21549^T with the *Entercococcus*-specific primers Ent 1 and Ent 2 (Ke et al., 1999) (B).

First line of nucleotide sequences are 16S rRNA gene sequence or tuf gene sequences of strain DSM 21549^T and second line the analyzed primer sequence. Numbers indicate base pair positions and dots coincident binding of bases.

We relied on the proposed guidelines for antibiotic susceptibility testing set out by Klare et al. (2005; 2007) for lactic acid bacteria since such tests were not standardized as yet for *Vagococcus* species. Dilution methods provided reliable MIC values for phenotypic antibiotic resistance profiling and disc diffusion inhibition zones were helpful for first indication of antibiotic resistance. Phenotypic trimethoprim resistance in strain DSM 21459^T could be attributed to a *drf*(G) resistance gene. Phenotypic tetracycline resistance in this strain could not be assigned to the 14 tetracycline resistance genes tested withthe microarray assay (Perreten et al. 2005). We assume therefore that tetracycline resistance is either induced by resistance genes not yet included in the microarray assay or is conferred by an as yet unidentified mechanism.

Surprisingly, strain DSM 21459^T tested positive in both the enterococci-specific PCR assays based on 16S rRNA gene sequence (Deasy et al. 2000) or *tuf* gene (Ke et al. 1999) sequences (data not shown). Strain DSM 21459^T would be incorrectly identified with this methodology as a member of the genus *Enterococcus.* These PCR assays were compared with primer homology to 16S rRNA gene and *tuf* gene sequences of strain DSM 21459^T (Figure 16). Primer E1 (Deasy et al. 2000) mismatched at its 8th and 12th position with the 16S rRNA gene sequence of strain DSM 21459^T (Figure 16A) and primer Ent 2 (Ke et al. 1999) at its 16th position on the *tuf* gene sequence of strain DSM 21459^T (Figure 16B). In both PCR setups one primer has no mismatch and the second primer had a single or two mismatches which should theoretically not permit PCR amplifications.

Ten as yet unidentified isolates from spontaneous *fènè* fermentation processes were typed to the novel *Vagococcus* species with the novel developed species-specific PCR, described herein. They were grouped into four different strains with rep-PCR, showing different banding patterns.

These strains displayed genotypic and phenotypic inter-species differences whereof lactose fermentation by strain CM-21 is exceptional for *Vagococcus* species and can probably be related to different plasmid profiles found within the four strains of DSM 21459^T.

We suggest that DSM 21459^T represents the type strain among four strains of the newly proposed *Vagococcus* species, *Vagococcus teuberi* sp. nov. based on results of the conducted polyphasic typing approach.

7.7 Description of *Vagaococcus teuberi* sp. nov.

Vagococcus teuberi (teu.ber.i M.L. gen. n. *teuberi* of Teuber), for the dairy and food microbiologist Prof. Dr. Michael Teuber, emeritus.

Cells are Gram-positive and catalase-negative diploid cocci with pili-like extensions on the cell surface, occurring in pairs or short chains. Cells are elongated in the direction of the chain. Good growth occurs in MRS-glucose and in TSYE medium at 30-37℃ at a μmax of 0.92/h. Bacteria grow on TSYE agar plates in white, round colonies with 1 mm in diameter, when incubated at 37℃ for 18 h. The colony surface and margins are smooth. Limited growth without proteolytic activity was observed in milk. Minimal and maximal growth temperatures in TSYE medium are 10℃ and 41℃, respectively. Cells grow, also in the presence of 6.5% NaCl, at pH 4.7 and pH 9.0 but not at pH 9.6. No gas is produced when grown in MRS-glucose. Strain DSM 21459T exhibits γ-haemolysis on Columbia blood agar, is a facultative anaerobe and will not tolerate 0.04% tellurite. Serogrouping is negative on antigens A, B, C, D, F and G. Acid is produced from ribose, trehalose, saccharose, maltose and methyl-β-D-glucopyranoside, but not from mannitol, sorbitol, lactose, raffinose, L-arabinose, D-arabitol, cyclodextrin, glycogen, pullulane, melibiose, melezitose or tagatose. Enzymatic activities were detected for β-glucosidase, pyroglutamic acid arylamidase and glycyl-tryptophan arylamidase. Arginine dihydrolase, β-galactosidase, β-glucuronidase, alkaline phosphatase, alanyl-phenylalanine-proline arylamidase, N-acetyl-β-glucosaminidase, β-mannosidase and urease activities were not detected. Acetoin production tested negative and hippurate was not hydrolyzed. Oleic acid, palmitic acid and stearic acid are the predominant cellular fatty acids. The G+C content of the DNA is 33.9 mol%. The type strain is DSM 21459^T which was isolated from the spontaneously fermented sour milk *fènè* at household level in Kasséla, Mali. Four different strains were identified with a novel species-specific PCR and further defined by rep-PCR.

7.8 Acknowledgments

We acknowledge the inputs to this study from Zakaria Farah (ETH Zurich, Switzerland), Jakob Zinsstag (Swiss Tropical Institute, Basel, Switzerland) and Saïdou Tembely (Laboratoire Central Vétérinaire (LCV), Bamako, Mali). Aminata Sissoko-Thiam and Idrissa Traoré (LCV) assisted in the isolation of microbes. Electron micrographs were taken by Stephan Handschin (ETH Zurich) and Vincent Perreten (Institute of Veterinary Bacteriology at the University of Bern) was highly involved into executing microchip hybridization assay for genotypic antibiotic resistance profiling. This project was financially supported by ETH Zurich internal funds and the Walter Hochstrasser Foundation (Zurich, Switzerland). We also acknowledge the Swiss national Centre of Competence in Research (NCCR) North–South for providing the research platform support through the Institute of Sahel.

8 Conclusion and outlook

The first hypothesis of this study was that environmental microbiota induces spontaneous *fènè* fermentation process through post-heat treatment contamination and that bacterial strain diversity depends on seasons due to the different temperature conditions of the cold, hot and rainy seasons.

We found only four strains of the highly diverse microbiota of 126 identified strains, in more than one season. Significantly lowest bacterial counts in *fènè* microbiota were detected during rainy seasons, whereas counts for hot and cold seasons were similarly elevated. Bacterial counts analyzed during the first four hours of spontaneous *fènè* fermentation process from raw milk were significantly highest in the hot season. The complex spontaneous *fènè* fermentation process was initiated by streptococci and enterococci colonizing cooling milk after heat treatment as first bacterial genera. Strain diversity fluctuated from fermentation to fermentation and could be traced to environmental contaminants with five strains detected simultaneously on the surface of the fermentation vats / the surrounding air and the corresponding fermentation processes. Most of the strains were only detected once during the fermentation process indicating the need for more extensive multiple sampling to understand strain dynamics during the spontaneous *fènè* fermentation process in more detail.

The second hypothesis was that uncontrolled antibiotic use, recogonized as a problem in developing countries, induces a selective pressure on microbiota and favors antibiotic resistance in bacteria as well as leads to a gene pool of transferable antibiotic resistance genes in *fènè.* Accurate typing of selected isolates is needed to address this question and through subsequent development of adapted starter cultures from well-typed isolates the third hypothesis was generated: Potential human health threats are reduced by the application of a safe adapted starter culture.

High bacterial counts of 10^7 – 10^9 cfu/ml in ready-to-eat *fènè* for presumptive enterococci, *Enterobacteriaceae* and over 10^5 cfu/ml *Staphylococcus aureus* indicated a serious hazard to human health. This threat was underscored by the finding that over 60% of the more than 1500 Gram positive and catalase negative bacteria isolated from *fènè* microbiota were enterococci and streptococci. Surprisingly no *St. thermophilus* strain was found among the streptococci but *St. infantarius* subsp. *infantarius* was identified instead as main acidifier in spontaneous *fènè* fermentations. *St. infantarius* subsp. *infantarius* is known in human medicine to cause severe bacteremia and

endocarditis and is strongly related to colonic and non-colonic cancer (Corredoira et al. 2008a; Corredoira et al. 2008b) and should therefore be banned from food. *St. infantarius* subsp. *infantarius* however should be analyzed in detail on its pathogenicity thus there is some evidence that it even might be used once as a fermentative species. In this light *St. gallolyticus* subsp. *macedonicus,* which also belongs to the *St. bovis* / *St. equinus* complex (Schlegel et al. 2003) is proposed as an adjunct starter culture for cheese fermentation (De Vuyst and Tsakalidou 2008) and the innocuous *St. thermophilus* emerged and evolved by combination of loss-of-function and horizontal gene transfer events as sole and for the dairy industry irreplaceable species among the other commensal and opportunistic streptococci species (Delorme 2008).

Strains of *St. infantarius* subsp. *infantarius* isolated in our study could not be considered for a possible fermentative applications since all strains analyzed were tetracycline resistant and harbored the tetracycline resistance gene *tet*(S). Tetracycline and even multi-drug resistant *St. infantarius* subsp. *infantarius* and *St. gallolyticus* subsp. *macedonicus* strains were isolated in an ancillary project from sour milks bought in Kenya, Somalia and the periphery of Bamako. Oxytetracycline is the antibiotic of first choice in the Malian agriculture and is also applied in human medicine. We found an extensively tetracycline resistance gene pool in *fènè* with the tetracycline resistance genes *tet*(S), *tet*(L) and *tet*(M) being present among different genera of LAB. The tetracycline and oxytetracycline resistance prevalence among enterococci was related to the assumed environmental oxytetracycline pressure applied and was clearly highest with 40% during the cold season. 17% of non-*Enterococcus* LAB intended for the development of adapted starter cultures were resistant against tetracycline.

There are several ways to address the antibiotic resistance problems related to the consumption of *fènè.* Antibiotic use and withdraw times should be well regulated and controlled, however this is not feasible within short time periods due to limited veterinary support available to farmers and the very low milk production prohibiting farmers to withdraw milk from the market. A more workable option would be to heat *fènè* prior to consumption; however this would clearly change sensorial properties of *fènè* if *fènè* is eaten as yogurt like sour milk. The most appropriate approach to eliminate antibiotic resistant bacteria from *fènè* microbiota would be to implement controlled *fènè* production by applying a safe and adapted starter culture and rigorous hygienic measures.

We developed a safe and adapted starter culture for small-scale *fènè* production with bacteria isolated from traditional *fènè* microbiota. This starter culture consists of five

different species, whereof *St. infantarius* subsp. *infantarius* was replaced by a commercial *St. thermophilus* starter culture. The industrial starter culture might once, after more research work be replaced again with a good and safe acidifier isolated from the traditional *fènè* microflora. The starter culture exhibits high stability and fast acidification at 35℃ and 40℃ and includes a bacte riocin producing *Lb. plantarum* strain inhibiting the growth of *L. ivanovii* HPB28 and *E. faecalis* DSM 2048^T. Finally small-scale *fènè* production was evaluated with the novel starter culture, the industrial *St. thermophilus* starter culture and traditional spontaneous fermentation at local conditions at ambient temperature of 27℃ – 36℃. Significantl y fastest acidification was achieved by the novel starter culture compared to the industrial starter culture and spontaneous fermentation. Post-heat treatment contamination by opportunistic pathogens could not be eliminated due to the application of starter cultures but was reduced from approximately 10^6 cfu/ml to 10^3 cfu/ml compared to traditional *fènè* fermentations. Preliminary sensorial testing with local consumers indicated a high acceptance of *fènè* produced with the novel starter culture, whereas *fènè* produced with the industrial *St. thermophilus* starter culture displayed too ropy consistence for local taste.

Our final hypothesis was that it should be possible to detect a novel species by the analysis of *fènè* microbiota as an unknown microbiological source. We identified among 217 unidentified Gram positive catalase negative bacteria 10 isolates belonging to four strain clusters of the novel species *Vagococcus teuberi* sp. nov. by a novel developed PCR approach amplifying a 207 bp fragment of the 16S rRNA gene sequence. *Vagococcus* species have not been found in milk and are therefore not listed in the EFSA list of microorganisms suitable to use in dairy products. The role of *Vagococcus teuberi* sp. nov. in milk fermentation due to its inability of lactose fermentation and the absence of proteolytic activity as well as its pathogenic properties are not known.

It would be great to benefit in the near future from this new knowledge and implement starter culture application at small-scale sour milk production in Africa and also to initiate local starter culture application. It should be analyzed in more detail if *St. infantarius* subsp. *infantarius* displays a serious health threat to human or if strains isolated within this work might be suitable for novel fermentative application. A further research project could include extended antibiotic resistance profiling, analyzing the link between antibiotic pressure applied in local animal husbandry and the transfer of antibiotic resistant bacteria to human beings through the agro food chain as well as the establishment of antibiotic resistance gene pools along the agro food chain. Finally it

would be helpful to develop molecular typing techniques to identify the genus *Vagococcus* and to discriminate its species, investigate technological or pathogenic properties of *V. teuberi* and to elucidate the reason for its phenotypic tetracycline resistance, which could not be related to a known acquired tetracycline resistance gene.

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Curriculum Vitae

Stephan Markus Wullschleger

Born February 4, 1980

Citizen of Aarburg (AG)

Switzerland

2005 – 2009	ETH Zurich, Switzerland Scientific collaborator and assistant, PhD thesis
2004	Diploma degree in Food Science and Nutrition
1999 – 2004	Studies in Food Science and Nutrition at ETH Zurich
1999	Matura type C
1995 – 1999	Kantosschule, Sargans