Acta agriculturae Slovenica, 90(december 2007)2, 107–113.

http://aas.bf.uni-lj.si

Agris category codes: L51 COBISS Code 1.01

EXAMINATION OF AI-2 QUORUM SENSING SYSTEM IN *Prevotella bryantii* AND *Prevotella ruminicola*-LIKE STRAINS BY USING BIOLUMINISCENCE ASSAY

Gregor GORENC [a)], Filip LUKÁŠ [b)] and Gorazd AVGUŠTIN [c)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Zootechnical Dept., Chair for Microbiology and Microbial Biotechnology, Groblje 3, SI-1230 Domžale, Slovenia.
[b)] Academy of Science of the Czech Republic, Institute of Animal Physiology and Genetics, Vídeňská 1083, 142 20 Prague, Czech Republic.
[c)] Same address as [a)], e-mail: gorazd.avgustin@bfro.uni-lj.si.

Received November 12, 2007, accepted December 03, 2007.
Delo je prispelo 12. novembra 2007, sprejeto 03. decembra 2007.

ABSTRACT

Rumen is an example of a complex microbial ecosystem where extensive intraspecies as well as interspecies cell-cell cross-communication is expected, but little is known about. Four ruminal *Prevotella* strains, *Prevotella bryantii* $B_1$4, TC1-1 and TF1-3, and *P. ruminicola*-like strain 223/M2/7A were examined for the presence of AI-2 type quorum sensing systems. *Vibrio harveyi* BB170 autoinducer bioassay was used in order to detect the production of AI-2 autoinducer in examined strains. Optimization of autoinducer bioassay was made to test the influence of sampling time and the substrate used in chemically defined medium CD on reporter strain luminescence signal. We discovered that AI-2 type qurum sensing system is present in *P. ruminicola*-like strain 223/M2/7A, which induced more than 74% of reporter strain bioluminescence, regardless of the presence of glucose in chemically defined medium CD. Such induction of bioluminescence was not observed in *P. bryantii* strains, however.

Key words: microbiology / rumen / *Prevotella* / quorum sensing / bioassay / *V. harveyi* BB170

UGOTAVLJANJE SISTEMA ZA ZAZNAVANJE KRITIČNE CELIČNE GOSTOTE AI-2 PRI BAKTERIJI *Prevotella bryantii* IN SEVIH, KI SO PODOBNI BAKTERIJI *Prevotella ruminicola*, S TESTOM BIOLUMINISCENCE

IZVLEČEK

Vamp prežvekovalcev predstavlja kompleksen mikrobni ekosistem, kjer pričakujemo obsežne tako znotrajvrstne kot tudi medvrstne medcelične komunikacije, vendar je o njih le malo znanega. Pri štirih vampnih sevih iz rodu *Prevotella*, t.j. *Prevotella bryantii* $B_1$4, TC1-1 in TF1-3 ter *P. ruminicola*-podobnem sevu 223/M2/7A smo preverili prisotnost sistemov za zaznavanje celične gostote tipa AI-2. Z uporabo biološkega testiranja avtoinduktorjev s testnim organizmom *Vibrio harveyi* BB170 smo ugotavljali produkcijo molekul AI-2 pri preučevanih mikroorganizmih. S preverjanjem vpliva časa biološkega testiranja ter substrata, uporabljenega v kemijsko definiranem gojišču CD, na signal bioluminiscence reporterskega seva, smo optimizirali biološko testiranje avtoinduktorskih molekul. Ugotovili smo, da je AI-2 tip sistema za medcelično komunikacijo prisoten pri *P. ruminicola* podobnem sevu 223/M2/7A, ki je induciral več kot 74 % bioluminiscence reporterskega seva, ne glede na prisotnost glukoze v kemijsko definiranem gojišču CD. Indukcije bioluminiscence pri sevih vrste *P. bryantii* nismo opazili.

Ključne besede: mikrobiologija / vamp / *Prevotella* / zaznavanje celične gostote / biološko testiranje / *V. harveyi* BB170

INTRODUCTION

Rumen is a complex ecosystem with mixed microbial community of high density and complexity, a semi-closed environment with many possible symbiotic and pathogenic conditions, where extensive possibilities, systems and routes of intraspecies as well as interspecies cell-cell communication could be found (Mitsumori *et al.*, 2003). Many bacteria use autoinducer molecules to monitor their population density, and regulate their gene expression and population behavior accordingly, a system termed quorum sensing. The first discovered type of quorum sensing system, AI-1, used by Gram-negative proteobacteria, employs acylated homoserine lactones for intra-species communication (Miller and Bassler, 2001). Recently it was reported that AI-1 was also present in rumen contents, but surprisingly, not in any of the cultured rumen bacteria (Erickson *et al.*, 2002). The second type of quorum sensing system, AI-2, is the most widely found quorum sensing system, used for inter-species communication. It employs short peptides, produced by LuxS homolog (Surette *et al.*, 1999; Bassler and Losick, 2006). The presence of AI-2 in rumen was also detected. Possible quorum sensing was reported in several rumen species, results interestingly depending on the type of the medium used in autoinducer bioassay (Mitsumori *et al.*, 2003).

Different substrates used, especially glucose, can significantly affect the outcome of AI-2 bioassay, even at low concentrations (DeKeersmaecker and Vanderleyden, 2003; Turovskiy and Chikindas, 2006). Modification and optimization of the protocol is important for the reproducibility and reliability of quorum sensing bioassay (Vilchez *et al.*, 2007). We have tested the influence of sampling time and the substrate used in chemically defined medium CD on reporter strain luminescence signal, and in this paper we report on our examination on four ruminal *Prevotella* strains, *Prevotella bryantii* $B_1$4, TC1-1 and TF1-3, and *P. ruminicola*-like strain 223/M2/7A for the presence of AI-2 type quorum sensing systems.

Strictly anaerobic Gram-negative bacteria from the genus *Prevotella* represent one of the most abundant and thus major bacterial populations within ruminant gut. They may constitute up to 70% of the culturable bacterial population from the ruminal samples (Van Gylswyk, 1990). The abundance of these bacteria in rumen fluid was confirmed also by several molecular enumeration studies (Wood et al, 1998; Tajima *et al.*, 2001; Reilly *et al.*, 2002; Stevenson and Weimer, 2007).

MATERIAL AND METHODS

Bacterial strains and culture conditions used

Four strictly anaerobic ruminal strains from genus *Prevotella* were used in this study: *P. bryantii* $B_1$4, *P. bryantii* TC1-1, *P. bryantii* TF1-3 and *P. ruminicola*-like strain 223/M2/7A. The origin of the strains was described previously (Avguštin *et al.*, 1997). Strains were grown in chemically defined media CD, described by Scott and Dehority (1965). 0.4% (w/v) glucose, 0.4% (w/v) cellobiose or 0.4% (w/v) of each glucose and cellobiose were used as a substrate. Bacterial strains were incubated in 10 ml of CD medium under anaerobic conditions at 39 °C for 1–2 days until maximum growth. The cultures were then separated into bacterial cells and culture supernatants by centrifugation at 10.000 x g for 15 min at 4 °C. Cell-free supernatants were stored at –20 °C until use in the autoinducer bioassay.

Two strains of *V. harveyi* (obtained from ATCC) were used in AI-2 autoinducer assay. *V. harveyi* BB170 (sensor 1^-, sensor 2^+) was used as the reporter strain and *V. harveyi* BB152 (AI-1^-, AI-2^+) was used as the producer of AI-2 molecules. Strains were grown overnight in AB medium (Greenberg *et al.*, 1979). Cell-free supernatant of *V. harveyi* BB152 was obtained in the same way as from *Prevotella* strains, and served as the positive control.

Autoinducer bioassay

The bioassay for detection of AI-2 molecules, reported by Surette and Bassler (1998) was used. Optimizations of the protocol were made as suggested by Vilchez *et al.* (2007), and DeKeersmaecker and Vanderleyden (2003). *V. harveyi* BB170 reporter strain was grown overnight at 30 °C in AB medium until optimum optical density of 1.0–1.1 at 600 nm was reached. The culture was then diluted 1:5000 in fresh AB medium to obtain 10^5 CFU ml^{-1}. 1 ml of the cell-free supernatant from tested ruminal strains, *V. harveyi* BB152 (positive control) or sterile CD medium (negative control) was added to 9 ml of diluted reporter strain, thoroughly mixed and then incubated at 30 °C with aeration (175 RPM). Within next 6 hours every hour a sample of bioassay mixture was retrieved, 5-fold diluted in 2% NaCl solution and the stimulation of light production in *V. harveyi* BB170 reporter strain was measured by luminometer (LKB Wallac 1251).

RESULTS AND DISCUSSION

As reported previously, AI-2 production is growth-medium dependent (Burgess *et al.*, 2002). Media composition can greatly influence the growth and luminescence of *V. harveyi* reporter strain BB170, as samples of the tested cultures are added to reporter strain in relatively large portion, i.e. 10% of the total volume. The presence of residual glucose in medium even at low concentrations (2 mM) completely inhibits the luminiscence of reporter strain (DeKeersmaecker and Vanderleyden, 2003). Nevertheless, glucose enables the reporter strain to grow faster than glycerol, which is the only carbon source in AB medium. It was shown that the presence of glucose in AB medium at 1.25 g/l can promote up to 7-times faster growth, causing higher densities of reporter strain and self-response during the bioassay. Bioluminiscence should be detected in response to the added AI-2 only at lower cell densities (no larger than 10^6–10^7 CFU ml^{-1}) of reporter strain, with the concentration of glucose below the level of inhibition (Turovskiy and Chikindas, 2006).

As reporter strain produces and senses its own autoinducer molecules, the time of the assay is of crucial importance. The measurement at the time with the most pronounced difference between positive and negative controls is considered the most informative. Our experiments confirm (Figure 1) that this difference is maximal 5 hours after inoculation, when interference by endogenous AI-2 of the reporter is less than 3% of bioluminescence signal, regardless of the substrate used in chemically defined medium CD. The cell-free CD medium without added substrate was also submitted to bioassay. Compared to CD medium with added substrates, no significant differences of luminiscence in reporter strain were observed, therefore indicating that the concentration of residual glucose in growth medium was below the level of bioluminescence inhibition.

All *P. bryantii* strains reached maximum growth rapidly, with optical density of more than 1.6 at 600 nm after only 18 h of incubation, whereas *P. ruminicola*-like strain 223/M2/7A achieved maximum growth more slowly, after 40–64 h (data not shown). Only after population transitions into the stationary phase, the concentration of AI-2 emerges at its peak (Turovskiy and Chikindas, 2006). The level of AI-2 then remains relatively constant all throughout the stationary phase with less than 1% change during 27 hours after entering the stationary phase (Surette and Bassler, 1998). The time of collecting the tested cell-free culture samples is therefore of less importance, provided that cultures had reached the stationary phase.

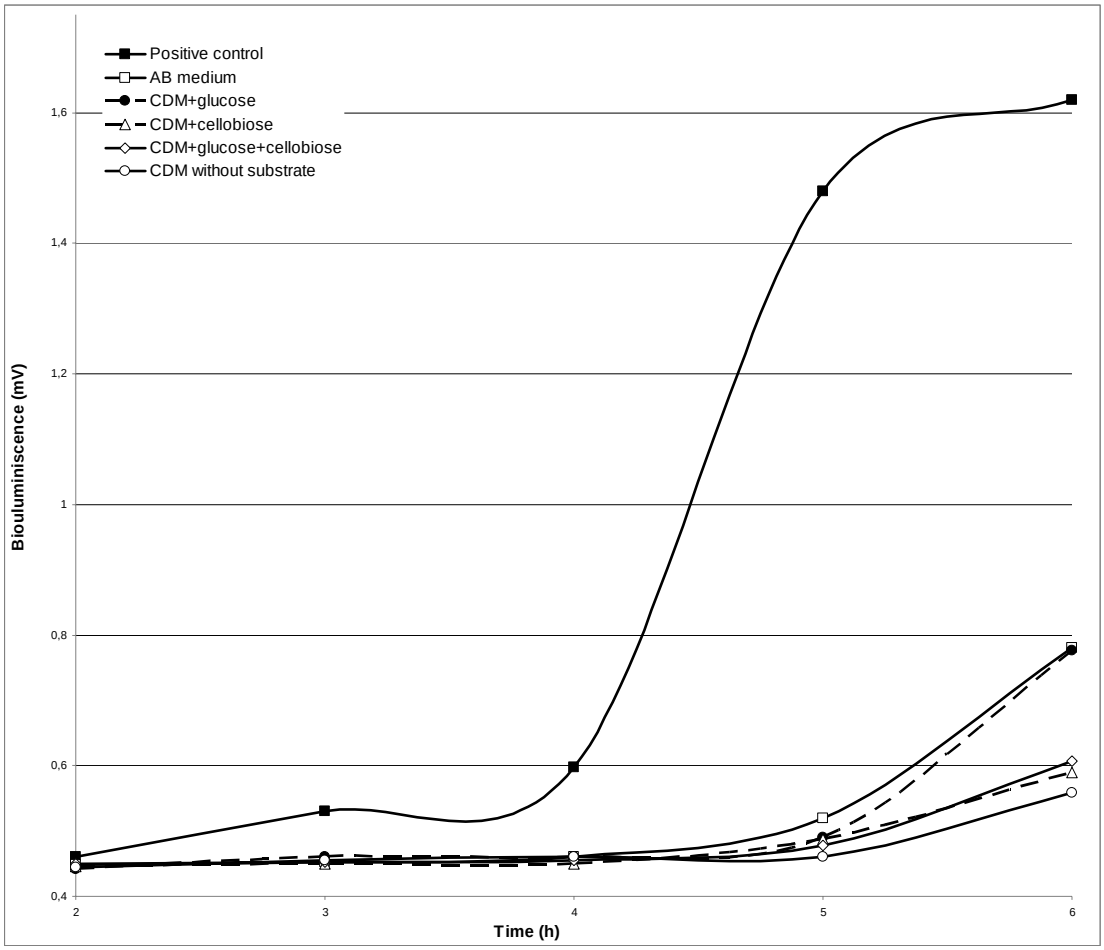

Figure 1. Determination of optimal time for autoinducer bioassay, using different media and substrates. Data is shown in absolute values of luminometer measurements. Samples were retrieved every hour during 6 hours of observation. Bioluminescence of the positive control grows slowly during the first 4 hours, after which it starts to peak rapidly. Bioluminescence of the samples from chemically defined medium CD (CDM) with different added substrates remains at the same level during the first 4 hours, and then starts to grow gradually due to the endogenously produced AI-2 of the reporter strain. The largest difference between bioluminescence measurements of positive control and the samples from chemically defined medium CD emerges 5 hours after inoculation.

Slika 1. Določanje optimalnega časa biološkega testiranja ob uporabi različnih gojišč in substratov. Prikazane so absolutne vrednosti meritev z luminometrom. Vzorci so bili odvzeti vsako uro, skupaj 6 ur. Bioluminiscenca pozitivne kontrole počasi raste prve 4 ure, nato pa se začne hitro povečevati. Bioluminiscenca vzorcev iz kemijsko definiranih gojišč CD (CDM) z dodanimi različnimi substrati praktično ne narašča do 4. ure, potem pa se postopoma povečuje zaradi nastajanja endogenih avtoinduktorskih molekul tipa 2 reporterskega seva. Največja razlika med bioluminiscenco pozitivne kontrole in vzorcev iz kemijsko definiranih gojišč CD se pojavi po 5 urah rasti.

Using the *V. harveyi* BB170 bioassay, considering culture cell density and growth time, we have clearly demonstrated the presence of AI-2 quorum sensing system in *P. ruminicola*-like strain 223/M2/7A, but not in any of the *P. bryantii* strains. *P. ruminicola*-like strain 223/M2/7A induced more than 74% of reporter *V. harveyi* BB170 bioluminiscence, regardless of the substrate used in chemically defined medium CD (Figure 2).

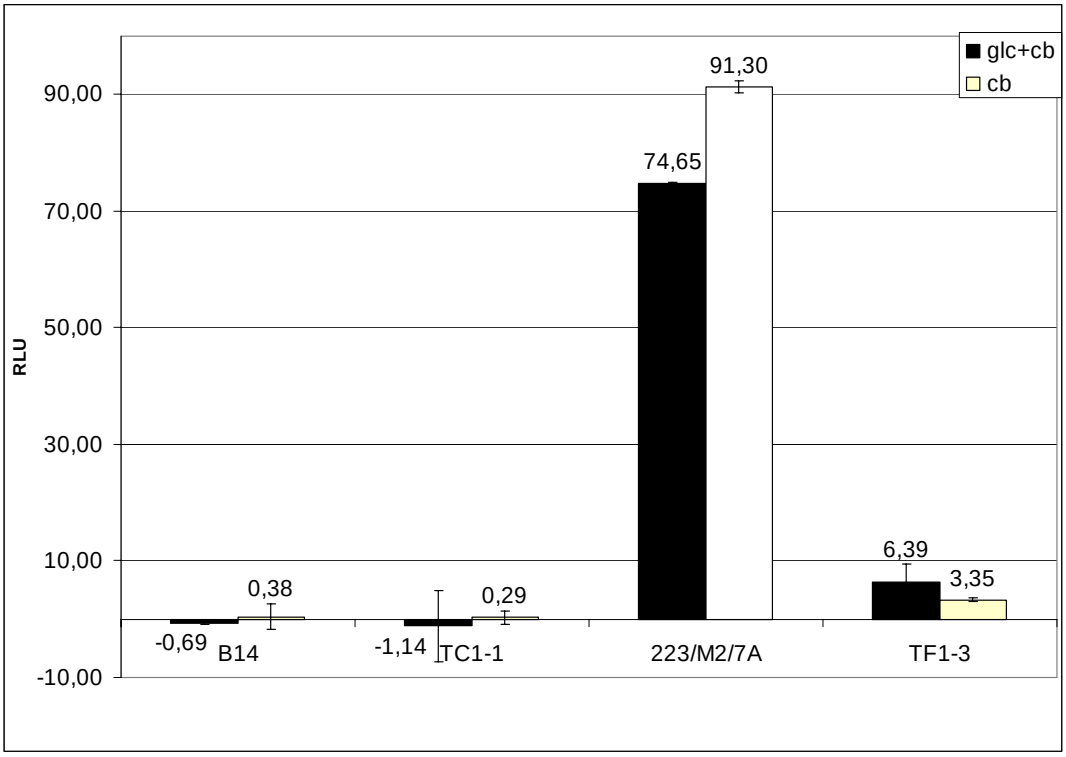

Figure 2. Average light induction in AI-2 bioassays of ruminal strains from the genus *Prevotella*. Bioluminiscence values are shown in relative light units (% induction of bioluminiscence in positive control). Black bars denote samples from chemically defined medium CD with added glucose and cellobiose as a substrate. White bars denote samples from chemically defined medium CD with added cellobiose as a substrate.

Slika 2. Povprečne vrednosti indukcije svetlobe pri biološkem testiranju avtoinduktorskih molekul tipa 2 pri sevih rodu *Prevotella* iz vampa. Vrednosti bioluminiscence so prikazane v relativnih enotah svetilnosti (delež indukcije bioluminiscence pri pozitivni kontroli v %). Črni stolpci označujejo vzorce iz kemijsko definiranega gojišča CD z dodano glukozo in celobiozo. Beli stolpci označujejo vzorce iz kemijsko definiranega gojišča CD z dodano celobiozo.

Partial substitution of glucose in some cases increases, in other oppositely decreases the induction of bioluminiscence in reporter strain, but never changes the outcome of bioassay. The increase in light production could be attributed to significant increase in growth rates of reporter strain due to addition of easily accessible substrate, as decrease could be caused by inhibition effect of glucose on bioluminiscence.

In *P. bryantii* $B_1$4, *P. bryantii* TC1-1 and *P. bryantii* TF1-3 stimulation of bioluminiscence, indicating the presence of quorum sensing system, was not observed. Despite *P. bryantii* strain TF1-3 induced significantly more light production in reporter strain than other *P. bryantii* strains, its 3.35–6.39% induction was below 10% induction threshold, sufficient for confirmation of AI-2 molecules (Bassler *et al.*, 1997).

We cannot affirm that the *P. bryantii* strains that didn't induce significant bioluminiscence of reporter strain do not possess quorum sensing systems of any type. In addition to the abovementioned factors that notably influence the autoinducer bioassay, the difference in autoinducer molecule structure between bacteria from the genus *Prevotella* and *V. harveyi*, two phylogenetically relatively distant bacteria taxons, could cause the inability for successful interspecies communication and consecutive failure to detect it by using the *V. harveyi* autoindicer bioassay. Alternatively, one could employ the chemical detection of AI-2 by HPLC

or GC, but at the present these methods are rarely used due to the instability and low concentrations of AI-2 molecules in biological samples (Vilchez *et al.*, 2007).

CONCLUSIONS

Our experiments confirmed that the signal of *V. harveyi* reporter strain is minimal 5 hours after inoculation, when interference by endogenous AI-2 of the reporter is less than 3% of bioluminescence signal, regardless of the presence of glucose in chemically defined medium CD. As it was expected, using *V. harveyi* BB170 bioassay, considering culture cell density and growth time, we have demonstrated the presence of AI-2 quorum sensing system in ruminal *Prevotella* strains. *P. ruminicola*-like strain 223/M2/7A induced more than 74% of reporter *V. harveyi* BB170 bioluminescence, regardless of the presence of glucose in chemically defined medium CD, however, such induction of bioluminescence was not observed in *P. bryantii* strains.

REFERENCES

Avguštin, G./ Wallace, J.R./ Flint, H.J. Phenotypic diversity among rumen isolates of *Prevotella ruminicola*, proposal for redefinition of *Prevotella ruminicola* and the creation of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. International Journal of Systematic Bacteriology, 47(1997), 284–288.

Avguštin, G./ Ramšak, A./ Peterka, M. Systematic and evolution of ruminal species of the genus *Prevotella*. Folia Microbiologica, 46(2001), 40–44.

Bassler, B.L./ Greenberg, E.P./ Stevens, A.M. Cross-Species Induction of Luminiscence in the Quorum-Sensing Bacterium *Vibrio harveyi*. Journal of Bacteriology, 179(1997), 4043–4045.

Bassler, B.L./ Losick, R. Bacterially speaking. Cell, 125(2006), 237–246.

Burgess, N.A./ Kirke, D.F./ Williams, P./ Winzer, K./ Hardie, K.R./ Meyers, N.L./ Aduse-Opoku, J./ Curtis, M.A./ Camara, M. LuxS-dependent quorum sensing in *Porphyromonas gingivalis* modulates protease and haemagglutinin activities but is not essential for virulence. Microbiology, 148(2002), 763–772.

DeKeersmaecker, S.C./ Vandrleyden, J. Constraints on detection of autoinducer-2 (AI-2) signalling molecules using *Vibrio harveyi* as a reporter. Microbiology Comment, 149(2003), 1953–1956.

Erickson, D.L./ Nsereko, V.L./ Morgavi, D.P./ Selinger, L.B./ Rode, L.M./ Beauchemin, K.A. Evidence of quorum sensing in the rumen ecosystem: detection of N-acyl homoserine lactone autoinducers in ruminal contents. Canadian Journal of Microbiology, 48(2002), 374–378.

Greenberg, E.P./ Hastings, J.W./ Ulitzur, S. Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. Archives of Microbiology, 120(1797), 87–91.

Miller, M.B./ Bassler, B.L. Quorum Sensing in Bacteria. Annual Review of Microbiology, 55(2001), 165–199.

Mitsumori, M./ Xu, L./ Kajikawa, H./ Kurihara, M./ Tajima, K./ Hai, J./ Takenaka, A. Possible quorum sensing in the rumen microbial community: detection of quorum-sensing signal molecules from rumen bacteria. FEMS Microbiology Letters, 219(2003), 47–52.

Reilly, K./ Carruthers, V.R./ Attwood, G.T. Design and use of 16S ribosomal DNA-directed primers in competitive PCRs to enumerate proteolytic bacteria in the rumen. Microbial Ecology, 43(2002), 259–270.

Scott, H.W./ Dehority, B.A. Vitamine requirement of several cellulolitic bacteria. Journal of Bacteriology, 89(1965), 1169–1175.

Stevenson, D.M./ Weimer, P.J. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Applied Microbiology and Biotechnology, 75(2007), 165–174.

Surette, M.G./ Bassler, B.L. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proceedings of the National Academy of Sciences of the United States of America, 95(1998), 7046–7050.

Surette, M.G./ Miller, M.B./ Bassler, B.L. Quorum sensing in *Escherichi coli, Salmonella typhimurium,* and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proceedings of the National Academy of Sciences of the United States of America, 96(1999), 1639–1644.

Tajima, K./ Aminov, R.I./ Nagamine, T./ Matsui, H./ Nakamura, M./ Benno, Y. Diet-dependent shifts in the bacterial population of the rumen revealed with real time PCR. Applied and environmental microbiology, 67(2001), 2766–2774.

Turovskiy, Y./ Chikindas, M.L. Autoinducer-2 bioassay is a qualitative, not quantitative method influenced by glucose. Journal of Microbiological Methods, 66(2006), 497–503.

Van Gylswyk, N.O. Enumeration and presumptive identification of some functional groups of bacteria in the rumen of dairy cows fed grass silage-based diets. FEMS Microbiology Ecology, 73(1990), 243–254.

Vilchez, R./ Lemme, A./ Thiel, V./ Schulz, S./ Sztajer, H./ Wagner-Döbler, I. Analysing traces of autoinducer-2 requires standardization of the *Vibrio harveyi* bioassay. Analytical and Bioanalytical Chemistry, 387(2007), 489–496.

Wood, J./ Scott, K.P./ Avguštin, G./ Newbold, C.J./ Flint, H.J. Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. Applied and Environmental Microbiology, 64(1998), 3683–3689.