DISEÑO DE UN MEDIO DE CULTIVO PARA EL AISLAMIENTO Y CRECIMIENTO DE LA BACTERIA PRODUCTORA DE HIDRÓGENO *Ruminococcus albus*

DESIGN OF A CULTURE MEDIUM FOR ISOLATION AN GROWTH OF THE HYDROGEN-PRODUCING BACTERIUM *Ruminococcus albus*

Diana Paola Sanabria Lozano

Universidad Nacional de Colombia

Facultad de Ciencias, Posgrado Interfacultades de Microbiología

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Director:
Carlos Alberto Guerrero Fajardo, Químico, MSc Ing. Ambiental Ph. D. Química
Codirector:
Jean-Pierre Magnin, Microbiólogo, Ph. D.

Colaborador:
Roderick Mackie, Microbiólogo Ph.D.

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A aquellas instituciones colombianas dedicadas al estudio de los microorganismos anaerobios y a los profesionales de las Ciencias de la Vida quienes se conmueven y dan rienda suelta a su curiosidad por estos microorganismos.

"No hay que ensillar antes de traer los caballos"
Adagio popular

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Hace 5 años cuando emprendí esta etapa de mi vida, estuve enfrentada a dos caminos y una decisión: cursar mi maestría en Colombia o fuera de Colombia. En ese momento veía partir a varios de mis compañeros de pregrado con la idea en la cabeza de cursar sus estudios de posgrado en el exterior y en lo posible trabajar afuera porque Colombia no ofrecía muchas posibilidades a quienes nos dedicamos a las ciencias. Yo elegí quedarme y cursar mi maestría en la Universidad Nacional de Colombia, entre otras cosas porque quería ampliar mi perspectiva acerca del contexto de las ciencias en mi país, empaparme de esta gloriosa *alma mater*. Quería conocer puntualmente cuales eran nuestras fortalezas, nuestras debilidades y adquirir nuevas habilidades y conocimientos. De esta manera, en el momento en que decidiera salir de mi país no lo haría huyendo de una desvalorización de mi profesión, sino para aprender, adquirir experiencia y compartir la propia en contextos diferentes. Sin embargo, nunca conté con que me enfrentaría con una serie de obstáculos que, aunque retrasaron y entorpecieron mi trabajo y mi experiencia, me han fortalecido y forjado como persona y profesional y que gracias a todos ellos, me siento preparada y madura para proseguir mi carrera profesional a un nivel superior en otro lugar del mundo. Sea donde sea que yo vaya y sea que regrese a mi país o no, quiero contribuir al desarrollo y posicionamiento de las Ciencias Biológicas. Tenemos una riqueza biológica inmensa en nuestro territorio que merece ser valorada, conservada y aprovechada de una manera sostenible y una calidad humana y talento profesional increíbles en nuestra gente. No esperemos a que sea tarde para lamentarnos por nuestra riqueza biológica mal aprovechada y nuestros cerebros fugados.

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Resumen

Ruminococcus albus es una bacteria fibrolítica que ejerce una función importante en el fluido ruminal por su capacidad para degradar celulosa y hemicelulosa y por sus interacciones con otras especies. Asimismo, es un microorganismo con alto potencial biotecnológico por su capacidad para producir hidrogeno y porque puede ser usado como complemento dietario de ganado, pollos y caballos. A pesar de que esta bacteria se ha estudiado desde 1950, la mejora de medios de cultivo que permitan su aislamiento selectivo y específico a partir de fluido ruminal contribuiría a facilitar su estudio y aplicabilidad. El objetivo de este estudio fue optimizar un medio de cultivo para su aislamiento y crecimiento evaluando diferentes medios y agentes selectivos. En una primera fase se evaluó el método de roll-tube para aislar microorganismos celulíticos propuesta por Hungate (1969), utilizando carboximetilcelulosa (CMC) y papel filtro Whatman N°1 (PMC) como fuentes de carbono en un medio modificado basado en el medio RGC (Rumen Fluid Glucose Cellobiose), identificando solo tres aislamientos de *Streptococcus bovis/equinus* en lugar de *R. albus*. En una segunda fase se evaluó el crecimiento de la cepa 7 de *R. albus* en el medio DSMZ 436 cuya reactivación tardó nueve días y los repiques crecieron después de 48 horas incubando a 37°C. Posteriormente se evaluó un medio químicamente definido, midiendo la tasa de crecimiento de las cepas 7, 8, SY3 y B199 que alcanzaron la fase exponencial tardía después de aproximadamente 10 horas de incubación a 37°C y 39°C. Adicionalmente se evaluó el crecimiento de las cepas en presencia de diferentes compuestos, con el fin de proponerlos como agentes selectivos. La cepa B199 creció después de 48 horas de incubación en presencia de ácido propiónico al 0.5% y LiCl al 0.5%, mientras que el resto crecieron antes de las 24 horas. Asimismo, todas las cepas crecieron en presencia de ácido nalidixico, exceptuando la cepa 8. Para estudios futuros se propone aislar *R. albus* usando fibra vegetal como fuente de carbono y probar la selectividad de estos tres agentes en el medio químicamente definido mediante la técnica de roll-tube y evaluar el crecimiento del microorganismo frente a otros posibles agentes selectivos como el feniletil alcohol entre otros. Del mismo modo se recomienda que para aislar, cultivar y criopreservar exitosamente este microorganismo es necesario tener en cuenta las características de su metabolismo y de su hábitat y seguir las recomendaciones sugeridas por la literatura para el trabajo en el laboratorio.

Palabras clave: *Ruminococcus albus*, bacterias celulolíticas, microorganismos ruminales

Abstract

Ruminococcus albus is a fibrolytic bacterium which develops a key role in the rumen fluid due to its cellulose and hemicellulose degrading capability and interaction with other rumen microbiota species. Furthermore, is a high-biotechnological potential bacterium able to be used to produce hydrogen as energy source and as a dietary complement for poultry, cattle and horses as well. Therefore, despite of *R. albus* has been a research matter from 1950, a culture medium to be specifically isolated from rumen fluid would open the possibilities of study and applicability. The aim of this study was to optimize a selective culture medium for its growth and isolation by evaluating different media and selective agents. In a first stage, roll-tube methodology for isolation of cellulolytic bacteria proposed by Hungate (1969) using carboxymethyl-cellulose (CMC) and pebble-milled cellulose (PMC) as carbon sources, was evaluated in a RGC-based (Rumen Fluid Glucose Cellobiose) culture medium. Three ruminococci-shape isolates were identified as *Strptococcus bovis/equinus* instead *R. albus*. In a second phase, growth of *R. albus* strain 7 was evaluated in the medium DSMZ 436, in which reactivation took nine days and fresh inocula took 48 hours to growth. Both incubations were carried out at 37°C. Afterwards a chemical-defined medium was evaluated measuring the growth rate of *R. albus* strains 7, 8, SY3 and B199 which reached the late exponential phase before 10 hours of incubation at 37°C and 39°C. Subsequently the growth of the four strains was evaluated in the presence of several compounds with the aim to propose it as selective agents. The strain B199 grew after 48 hours of incubating time in the presence of 0.5% propionic acid and 0.5% LiCl, meanwhile the remaining strains have grown yet at 24 hours of incubation. All strains grew in the presence of 25 µg/mL of nalidixic acid as well, but strain 8 didn't. For further studies it is proposed to isolate *R. albus* using vegetable fiber as carbon source and screening the selectivity of these agents in the chemical-defined medium by the roll-tube technique. However, the resistance to other selective agents of *R. albus* strains as phenyl-ethyl alcohol among others should be evaluated as well. Moreover, for a success isolation, growth and cryopreservation of *R. albus* it is recommended to take in consideration metabolic and habitat features of this microorganisms and follow the lab procedures as suggested in the literature.

Keywords: *Ruminococcus albus*, ruminal bacteria, cellulolytic bacteria

List of figures

Page

Figure 1: a) Electronic microscopic view of a *Ruminococcus albus* cell (1), attached to a cellulose fiver (2) through the cellulosome (3) (Curtesy of Fernando Rodriguez-Villamizar, 2012) **b)** Variable-pressure scanning electron micrograph of *R. albus 7* cells and associated glycocalyx adhering to cellulose fibers (CF) (1) Cells from young (24-h) cultures (2) showing discrete cellular appendages (arrows) (3), (Weimer *et al.,* 2006). 6

Figure 2: Mixed acid fermentation pathway. *Ruminococcus albus* produces ethanol, formate, acetate, and CO_2 from glucose and cellobiose derived from breaking down of cellulose. Succinate production has not been reported for this specie (blue dotted line). Some strains produce lactate (orange line). Depending on hydrogen partial pressure *R. albus* produces just hydrogen, acetate and CO_2, or these compounds in lower concentrations along with ethanol and tends to accumulate formate. Modified from Ntaikou and coworkers (2008). 7

Figure 3: 161 isolations were obtained from rumen fluid of five Colombian bovine breeds. a) Percentage of isolations recovered using CMC and PMC as carbon source. b) Percentage of isolations able to be cryopreserved after storage at 4°C, reinoculated and incubated at 39°C. 25

Figure 4. Morphologies of isolations obtained from rumen fluid using PMC and CMC independently as carbon source in a RGC-based culture medium. a) Vibrio shape rods of an isolation obtained from Harton del Valle individual in PMC medium. b) 'S' shape filaments of an isolate obtained from Romo sinuano individual in PMC medium. c) Spiral shape cells of an isolate obtained from Sanmartinero individual in CMC medium. d) Cocci obtained from Blanco orejinegro individual in CMC medium. e) And rods in chains obtained from Sanmartinero individual in PMC medium. 26

Figure 5. Morphology and Gram staining of cocci shape pure cultures recovered after cryopreservation. a) Isolate obtained from Blanco orejinegro individual in CMC medium. b) Isolate obtained from Romo sinuano individual in CMC medium. c) Isolate obtained from Romo sinuano individual in CMC medium. d) Isolate obtained from Costeño con cuernos individual in PMC medium. 26

Figure 6. Morphology and Gram staining of *R. albus* strain 7 cultures in the first trait for culturing this strain. a) First Gram staining. Both rods and cocci are observed. b) Morphology and Gram staining of the dilution dilution10^{-1} c) Morphology and Gram staining of the dilution 10^{-2}. Morphology and Gram staining of the dilution dilution10^{-5}. 29

Figure 7. Morphology and Gram staining of *R. albus* strain 7 cultures in the second trait for culturing this strain. a) First Gram staining which seems Gram-negative b) Second Gram staining which seems Gram-positive. 30

Figure 8. Morphology of *R. albus* strains at 24 hours of incubation at 37°C. a) strain 7, b) strain 8, c) strain SY3 and d) strain B199. 31

Figure 9. Growth curve of *R. albus* strains incubated at 37°C (green or blue line) and at 39°C (red line). a) Strain 7, b) strain 8, c) strain SY3 and d) strain B199. 33

Figure 10: Diagram which represents the isolations obtained by Nyonyo and coworkers (2014) grouped by their degradation capability of PMC, CMC and xylan. Several isolations are able to degrade two or even the three carbon sources. Modified from Nyonyo and coworkers (2014). 36

List of tables

Page

Table 1: *R. albus* strains screening resistance to selective agents after 24 hours of incubation. 32

Table 2: Components of culture media expressed in percentage used for *R. albus* grow traits. 39

Contents

Resumen/Abstract ..... **VII**

List of figures ..... **IX**

List of tables ..... **X**

Introduction **3**

1. Theoretical frame **5**
1.1 *Ruminococcus albus* in the rumen fluid 5
1.1.1 Cellulose breakdown 5
1.1.2 Mixed-acid fermentation pathway 6
1.2 *Ruminococcus albus:* biotechnological applications 8
1.2.1 Hydrogen as an alternative fuel and energy source 8
1.2.2 Albusin B: A Gram-negative inhibitor and digestive system health promoter bacteriocin 10
1.3 Methods for isolation of cellulolytic bacteria from rumen fluid 10
1.4 Previous works in Colombia 10
1.5 Reported antibiotic screenings for *R. albus* 11
1.6 Chemical agents and antibiotics to be evaluated as *R. albus* selective agents . 12

2. Materials and Methods **16**
2.1 Microorganisms 16
2.2 Culture media composition 17
2.2.1 RGC-based culture medium 17
2.2.2 DSMZ436 culture medium 17
2.2.3 Chemical-defined medium 18
2.3 Isolation and identification of *Ruminococcus albus* from rumen fluid 19
2.3.1 Animals 19
2.3.2 Roll-tube technique for isolation of cellulolytic bacteria 19
2.3.1 Morphological identification of microorganisms 20
2.3.2 Cryopreservation procedure 21
2.3.3 Molecular identification of presumptive isolations 21
2.4 Evaluation of DSMZ 436 culture medium 21
2.5 Evaluation of chemical- defined culture medium 22
2.5.1 Cryopreservation procedure 22
2.5.2 Molecular identification of presumptive strains 23
2.5.3 Growth curve of R. albus strains 7, 8, SY3 and B199 in the chemical defined medium 23
2.5.4 Evaluation of agents to improve culture medium selectivity 24

3. Results **25**
3.1 Isolation and identification of *Ruminococcus albus* from rumen fluid of individuals belonging to five bovine Colombian breeds 25
3.2 Evaluation of DSMZ 436 culture medium 28
3.3 Evaluation of chemical- defined culture medium 30
3.3.1 Molecular identification of presumptive strains 31

3.3.2 Growth curve of *R. albus* strains 7, 8, SY3 and B199 in the chemical defined medium 32
3.3.3 Evaluation of agents to improve culture medium selectivity 32

4. Discussion 35
4.1 Recommendations 37

5. Conclusions and future perspectives 46

6. References 48

7. Appendices 55
Appendix A: Opening of Ampoules and Rehydration of Dried Cultures (anaerobic microorganisms) recommendations of DSMZ (available in https://www.dsmz.de/) 55
Appendix B: protocol for genomic DNA extraction of rumen fluid samples and *R. albus* cells 57
Appendix C: protocol for PCR products DNA purification 59

Introduction

Ruminococcus albus is a non-spore forming Gram-positive strict anaerobic bacterium which lives in the ruminal tract and belongs to the order *Clostridiales*, family *Ruminococcaceae* (Hungate, 1966; Hungate, 1957). *R. albus* can be observed under the microscope as single cocci and by pairs. It can be biochemically identified by the presence of hydrogen, ethanol, formate, CO_2, and acetate in culture media as metabolic end-products. The unique protein family 37 CBM (carbohydrate-binding modules) has been reported to be only among *R. albus* strains and be responsible for cell attachment to carbohydrates (Dassa *et al.,* 2014; Ezer *et al.,* 2008; Xu *et al.,* 2004).

The importance to study *R. albus* relays on its intraspecific and interspecific relationships, and the impact on the rumen microbiome, and on several potential biotechnological applications. The rumen microbiome is a dynamic and complex ecosystem in terms of biodiversity, exhibiting both competitive and symbiotic kinds of relationship (Mizrahi, 2013) in which *R. albus* plays a key role. Furthermore, the study of ruminants' digestive system and microbiome has allowed to elucidate how does the transfer of energy and nutrients from plant polysaccharides to cattle food products works (meat and milk) (Flint *et al.,* 2008).

In this sense, metagenomics tools have allowed to increase the knowledge about rumen ecology dynamics as other environments as well. The number of reports of previous unknown microorganisms from different environments among them, rumen microbiome is higher than in previous years. For this reason, many researches have underestimated the use of culturing for microbiological study and considered it outdated. But other researchers have recently cultivated microorganisms previously considered "uncultivable", using strategies which intend to mimic the conditions of their natural environment which have open the field to the "culturomics". So the use of metagenomics tools in combination with culturomics strategies may complement quite well the study of these recently discovered microorganisms (Lagier *et al.,* 2015).

The first step to conduct a study about *R. albus* or any other bacteria it is very important to be able to well grow and preserve it, and if it is required, to be able to isolate. This would warranty the possibility to develop any research project. *R. albus* has been studied from 60

years ago but the only medium designed specific for this microorganisms was the one proposed by Taya and coworkers (1980) in which they suppress the use of rumen fluid, yeast extract and the tryptone, and minimize de mineral salts as well. The other culture media used for *R. albus* isolation and cultivation can be used to work with other rumen bacteria species. Atlas recommend on his book (2010) to use a medium with glucose, cellobiose, yeast extract and volatile fatty acids (VFAs) mixture among other components for *R. albus* growth but a selective culture medium for specific *R. albus* isolation from rumen has not been reported yet.

Researchers use to isolate *R. albus* strains from rumen fluid by isolating cellulolytic species using fiber-based material both as carbon source and selective pressure factor as described by Hungate (1969). The objective of this project was to determine an optimal culture medium for isolation, growth and maintenance of *R. albus* evaluating three culture media: a RGC-based medium, the DSMZ 436 culture medium and a chemical defined-medium, highlighting both the critical components of the medium and the key steps of the process of preparation of the medium and cryopreservation of the cells which are determinant in the isolation and culturing of this microorganism. Inhibitory agents such as antibiotics and others also were evaluated in order to propose selective agents for isolation of *R. albus*. As the hypothesis of the present work, it can be stablished that the isolation and growth of *R. albus* in a given culture medium depends on how similar is it to the natural environment (Rumen) in terms of chemical components that promote its selection and growth and physical conditions.

1. Theoretical frame

1.1 *Ruminococcus albus* in the rumen fluid

This microorganism lives in the digestive tract of herbivorous mammals and have been reported in abundance mainly in the rumen (Dehority, 1973), a microbial ecosystem characterized to be highly diverse and complex comprised by bacteria (10^{10} to 10^{11} per mL), protozoa (10^4 to 10^6 per mL), and fungi (10^3 to 10^6 per mL) (Hespell *et al.,* 1997). *R. albus, Ruminococcus flavefaciens, Fibrobacter succinogenes* and *Eubacterium cellulosolvens*, among other microorganisms degrades cellulose synergistically, and with bacteria such as *Butyrivibrio fibrisolvens* and *Bacteroides ruminicola*, degrades hemicellulose of plants fiber (Dehority, 1973). In this way, both the animal and the microbial community are provided by oligosaccharides such as cellodextrins, cellobiose, glucose, xylose and arabinose which are used as carbon sources (Lynd *et al.,* 2002). On the other hand, rumen microorganisms produce large amounts of short-chain fatty acids which are absorbed and used as energy source by the animal (Hobson and Stewart, 2012).

1.1.1 Cellulose breakdown

Vegetal biomass is composed by cellulose, hemicellulose and ligning which comprise plant dry weigh in different percentages depending on the kind and age. Cellulose configuration consists of glycosil subunits linear chains which form elementary fibrils which aggregate by hydrogen bounds to form microfibrills, and these are in turn attached by Van der Waals forces to form the familiar cellulose fibers. In vegetal fiber cellulose is embedded in other polymers such as hemicelluloses, pectins and lignin (Lynd *et al.,* 2002).

R. albus degrades fiber through the cellulosome, a specialized extracellular complex proteic system (Figure 1.a). This complex is expressed under cellulose presence (Ohara *et al.,* 2000) and can be observed as a cell wall swelling embedded in the glycocalyx (Weimer *et al.,* 2006). Cellulosome works as an adherent to the substrate, trapping the oligosaccharides produced during cellulose degradation (Weimer *et al.,* 2006). *R. albus*

also sticks to the substrate and forms a one-cell layer biofilm trough fimbria-like structures (Figure 1.b) built by secretion system type IV (Rakotoarivonina *et al.,* 2002).

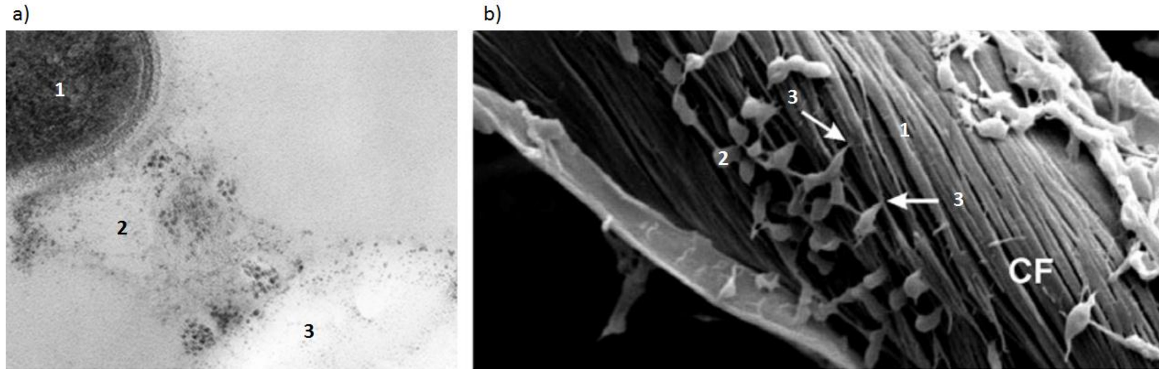
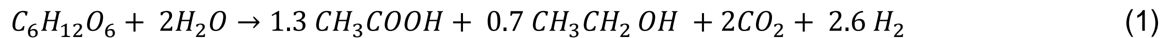


Figure 1: a) Electronic microscopic view of a *Ruminococcus albus* cell (1), attached to a cellulose fiver (2) through the cellulosome (3) (Curtesy of Fernando Rodriguez-Villamizar, 2012) **b)** Variable-pressure scanning electron micrograph of *R. albus 7* cells and associated glycocalyx adhering to cellulose fibers (CF) (1) Cells from young (24-h) cultures (2) showing discrete cellular appendages (arrows) (3), (Weimer *et al.,* 2006).

1.1.2 Mixed-acid fermentation pathway

In the cellulose degradation process*, R. albus* produces cellodextrin, cellobiose and glucose (Lou *et al.,* 1997) that are metabolized throughout the mixed-acid fermentation pathway (Figure 2) producing ethanol, hydrogen, carbon dioxide and short volatile fatty acids (Thurston *et al.,* 1993). Metabolic products and their related stoichiometry depend on culture conditions. For example, Iannoti and coworkers (1973) found that *R. albus* in batch culture produces from 1 mole of glucose, 1.3 mole of acetic acid, 0.7 mole of ethanol, 2 of CO_2 and 2.6 of H_2 (equation 1). Initial pH was 6.8.



$$C_6H_{12}O_6 + 2H_2O \rightarrow 1.3\, CH_3COOH + 0.7\, CH_3CH_2\,OH + 2CO_2 + 2.6\, H_2 \quad (1)$$

Besides when *R. albus* was cultured in a chemostat, fermenting 1 mole of glucose it produced values closed to 2 mole of acetic acid, 2 mole of CO_2, 4 mole of H_2 (equitation 2). The pH in the culture was maintained in 6.8 and gas was constantly removed by a peristaltic pump (Ntaikou *et al.,* 2009a). This stoichiometry is also feasible when *R. albus* is co-cultured with hydrogenotrophic microorganisms (Iannotti *et al.,* 1973).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2)$$

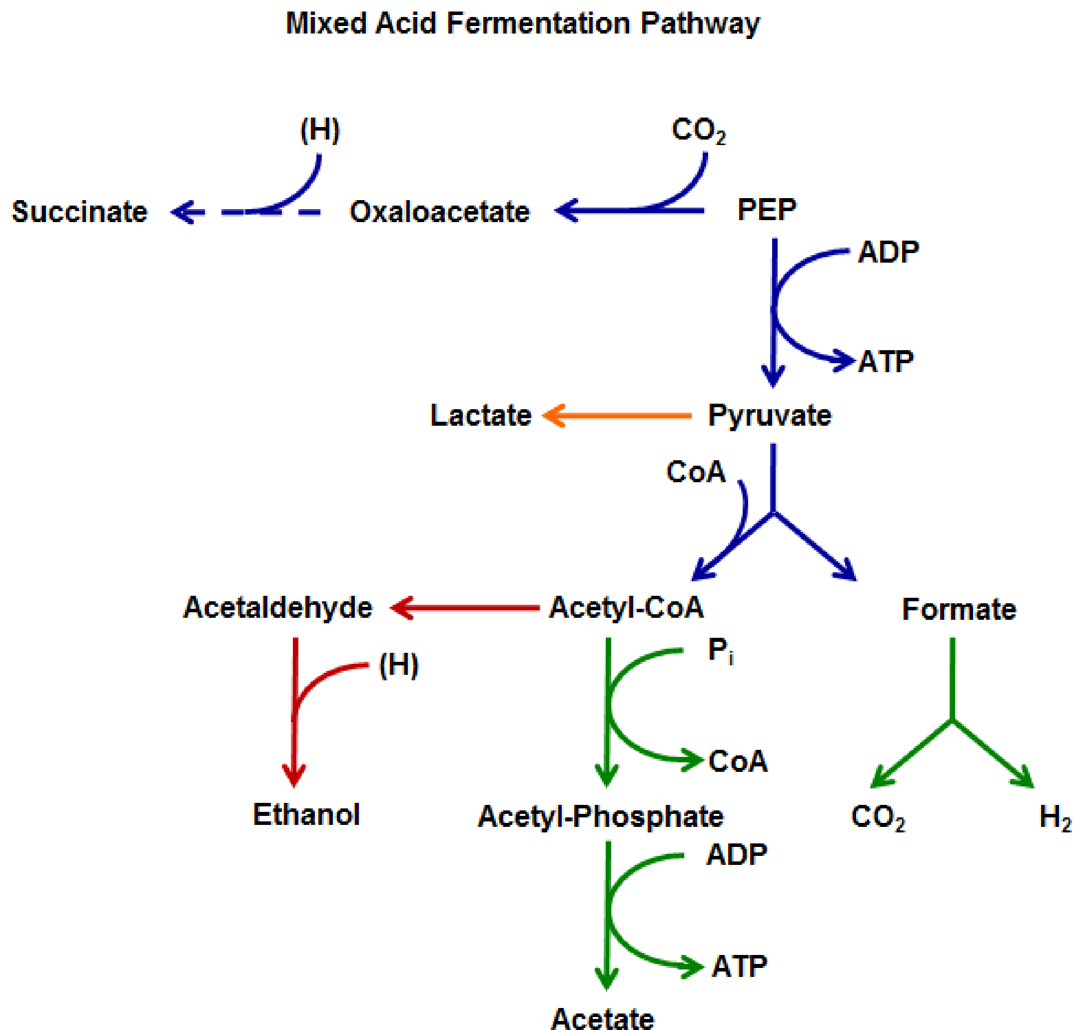


Figure 2: Mixed acid fermentation pathway. *Ruminococcus albus* produces ethanol, formate, acetate, and CO_2 from glucose and cellobiose derived from breaking down of cellulose. Succinate production has not been reported for this specie (blue dotted line). Some strains produce lactate (orange line). Depending on hydrogen partial pressure *R. albus* produces just hydrogen, acetate and CO_2, or these compounds in lower concentrations along with ethanol and tends to accumulate formate. Modified from Ntaikou and coworkers (2008).

This stoichiometric change can be explained as batch culture is a closed system while a chemostat is an opened system. When culture medium is inoculated and as time is passing, hydrogen partial pressure increases and pH value falls. Therefore, as hydrogen concentration increases in the atmosphere, its production in the cells tends to be thermodynamically unfavorable. Thus formate in the inner of the cells tends to accumulate and hydrogen production drops. In the same way, the half of the Acetil-CoA moles are going

to be transformed into acetaldehyde and further in ethanol, and the other half into acetate. Hence just 2 mol of ATP are going to be produced instead of 4 (Ntaikou *et al.,* 2009a).

On the other hand, the events related to hydrogen partial pressure in the rumen cavity are of interest in the *R. albus* and hydrogenotrophic species population dynamics. In rumen fluid it could be found syntrophic microorganisms which are removing the hydrogen produced by *R. albus* and by other hydrogen producing species continuously. For example, methanogenic and sulfate-reducing microorganisms. Therefore, it is expected hydrogen partial pressure in the rumen remains low in healthy ruminants. When hydrogen partial pressure increases, it seems hydrogen-consumers' metabolism is stimulated while, hydrogen-producers such as *R. albus* metabolism is disfavored (Stams and Plugge, 2009).

In the Ntaikou and coworkers (2009a) work, when *R. albus* was cultured in continuous at acid pH values (amongst 5.9 and 6.8), the stoichiometric proportions of the products were the same obtained in batch cultures by Iannoti and coworkers (1973) (equation 1). Ntaikou and coworkers (2009a) also evaluated the metabolites production at these low pH values combined with high hydrogen partial pressure. They noticed that ethanol and acetate proportions didn't change. So that, they concluded this stoichiometric change also occurs when external medium turns into acid as a mechanism of the cell to delay acidification because instead of 4 mol of acetate, 2 mol of acetate and 2 mol of ethanol are produced. In conclusion, for industrial process, in order to get an optimal cellulose degradation and hydrogen production by *R. albus*, the pH should be kept in neutral values (6.8-7.0) and a low hydrogen partial pressure should be maintained along the process.

1.2 *Ruminococcus albus:* biotechnological applications

1.2.1 Hydrogen as an alternative fuel and energy source

Due to traditional fuel and energy sources inconsistences related to its availability, economy, production, applications and environmental impacts, alternative energy sources and fuels are a key topic nowadays. Environmental impacts due to exploration and exploitation of traditional fuel and energy sources such as petroleum, and nuclear energy are well known (Demirbas, 2009). Additionally, for 2030 it has been estimated petroleum reserves would be able to supply the 75% of demands (Finley, 2012). So governments,

enterprises and the scientific community might work in mid-term projects in order to supply the remaining 25% or even a vast majority. Despite of fuels and energy marked depends on many variables and it makes the alternative sources massive entry to be uncertain, the work on them shall not stop.

Hydrogen is a suitable and attractive alternative source of energy and fuel for human activities such as transportation because of its high energetic content, cleanness and efficiency (Sharma and Ghoshal, 2015). Hydrogen can be produced by physicochemical and biological processes. Among each one of them biological are the cleanest ones but less efficient (Singh and Wahid, 2015). Nevertheless, by optimization of conditions and genetic engineering, it can be improved. Therefore, it is worth promoting the research on biological methods to produce hydrogen in the lab, and at a pilot and industrial scales and evaluate its commercial feasibility. So in the future it would be possible to ensure having disposable a very attractive energy and fuel source such as hydrogen.

Hydrogen has been the main *R. albus* biotechnological application of interest because in terms of quality and efficiency and based on the fact that the maximal theoretical performance is 4 mole of hydrogen per 1 mole of glucose (equation 2), and 8 moles per mole of cellobiose. This production can be reached by the bacterium maintaining it in a chemostat as was discussed before. Ntaikou and coworkers (2008) have reported that the *R. albus* strain 7 in batch cultures produces 2.76 mole of H_2 per mole of glucose (69% of performance), and per mole of cellobiose, 5.59 mole (69.87%). Additionally, in this study was found that *R. albus* produces 60L of hydrogen per kilogram of sorghum (*Sorghum bicolor*, and at 42 hours of hydraulic retention time in a chemostat, the 54% of the gas phase was hydrogen at the end of the process. In a further study conducted by the same research group (Ntaikou *et al.,* 2009b), the hydrogen production by *R. albus* was evaluated using different kind of papers: tissue paper, office paper, magazine paper, cardboard and newspaper. They found these corresponding values of hydrogen production per kilogram of each sort of paper: 282, 242, 94, 137 and 42. This is a higher production compared to sorghum, except for the newspaper. These differences of hydrogen production were due to paper composition such as the percentage of cellulose, carbohydrates and the lignin compounds and other cellulose metabolism-inhibitor substances.

1.2.2 Albusin B: A Gram-negative inhibitor and digestive system health promoter bacteriocin

Another biotechnological potential product of *R. albus* is the albusin B, a bacteriocin capable to inhibit *R. flavefaciens in vitro* (Chen *et al.,* 2004), and pathogen bacteria such as *Enterobacter aerogenes, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella enterica* and *Staphylococcus aureus* in poultry (Wang *et al.,* 2011). Furthermore, it has been demonstrated that albusin B increases *Lactobacillus sp.* counts in the feces of broiler chicken supplemented with recombinant yeasts expressing albusin B (Wang *et al.,* 2011). As a consequence, increasing in the absorption of nutrients in the small intestine and a gain in the body weight over control also were observed. Albusin B also increased the processing of phospholipids in the liver and strength the intestinal antioxidant defense as well (Wang *et al.,* 2013). In mice these benefits also have been observed (Hsieh *et al.,* 2013).

1.3 Methods for isolation of cellulolytic bacteria from rumen fluid

There are two methodologies to isolate cellulolytic microorganisms from rumen fluid: the “direct” and the “indirect” method (reviewed in McDonald *et al.,* 2012). The “direct” method developed by Hungate (1969) consists of preparing serial dilutions of fresh rumen fluid, inoculating dilutions 10^{-7}, 10^{-8} y 10^{-9} in rubber stopper tubes and rolling the agar. The selection agents in the tube are the anaerobiosis and the cellulose (filter paper Whatman N°1 or microcrystalline cellulose). After environ 48 and 72 hours of incubation at 39 °C is expected to have isolated cellulolytic colonies with clearance halos along the tube wall which belong to cellulolytic bacteria species of the rumen fluid (Hungate, 1969)., among them, *R. albus*. The “indirect” method consist in isolating a wide spectrum of bacteria in a general medium and then, evaluate the grown colonies for cellulolytic activity (Bryant and Burkey, 1953).

1.4 Previous works in Colombia

On the other hand, in Colombia few works in *R. albus* or even in *Ruminococcus* genus have been conducted. In 2003, Segura-Caro designed and evaluated a set of primers for

identification of *Ruminococcus* genus from rumen fluid of the Colombian breed of cattle "Blanco orejinegro". And recently (Rodriguez-Villamizar, 2014) the effect of *R. flavefaciens* in cattle was evaluated together with other rumen fluid species in a probiotic emulsion.

1.5 Reported antibiotic screenings for *R. albus*

Antibiotics screening for *R. albus* have been reported in previous studies. Champion and coworkers (1988) evaluated \the susceptibility of eight *R. albus* strains and nine *R. flavefaciens strains* to several antibiotics: ampicillin, bacitracin, chloramphenicol, clindamycin, erythromycin, kanamycin, losalocid, lincomycin, monencin, neomycin, penicillin, rifampicin, streptomycin and tetracycline. For both *R. albus* and *R. flavefaciens* they found that the inhibition halo was less than 0.1 cm using a concentration of 10g/mL of streptomycin. For the other antibiotics evaluated, for both species, some strains were resistant while others no. Therefore, streptomycin could be a good antibiotic candidate for the selective medium formulation. Streptomycin belongs to the aminoglycoside antibiotics that inhibits protein synthesis attaching to the 16S subunit of the ribosome of Gram-negative bacteria and some Gram-positive bacteria (Forge and Schacht, 2000). Below there are some aminoglycoside antibiotics postulated to be tested for *R. albus* resistance.

But in other study Tarakanov and Lavlinskii (2002) found different results. They also evaluated the susceptibility of 21 isolates of *R. flavefaciens* and 6 isolates of *R. albus* to the following antibiotics: tetracycline, ampicillin, erythromycin, streptomycin, rifampicin and chloramphenicol. As results for *R. albus*, they observed five of the strains were resistant to 10mg/L of erythromycin and three of these five were resistant to the same concentration of tetracycline, but just one of those three was resistant to the same concentration of streptomycin; another isolation which was resistant only to erythromycin was resistant to 10mg/L streptomycin as well. Despite in both mentioned studies, authors assured that the diet of the animals used for strains isolation were free of antibiotics. Results obtained in both studies does not permit to conclude which antibiotics *R. albus* resists and the resistance of some strains may were due to plasmid conference.

1.6 Chemical agents and antibiotics to be evaluated as *R. albus* selective agents

- pH:

It has been reported *R. albus* 7 is capable to degrade cellulose in a broad pH range: from 5.0 to 8.0, in contrast to its habitat partner species, *R. flavefaciens* C94 and *Fibrobacter succinogenes* A3c (Hiltner and Dehority, 1983). Morris (1988) found that the optimal pH values for cellulose adhesion of *R. albus* strains 20 and X3D54 were 5.5 and 7.0 (Morris, 1988).

- Propionic acid:

Propionic acid has been proposed as selective agent for bifidobacterial species recovery from feces. This compound inhibits species of *Enterobacteriaceae* and some Gram-positive bacteria such as *Enterococcus, Staphylococcus* and *Micrococcus* (Apajalahti *et al.,* 2003; Beerens, 1991).

- NaCl (sodium chloride):

Morris (1988) found that 0.5M and 2.5M NaCl increase adhesion to cellulose of the R*. albus* strains 20 and X3D54.

- LiCl (Lithium chloride):

Used as selective agent in Baird-Parker culture medium for isolation of Staphylococcus aureus. Inhibits enteric cocci and Gram-negative bacilli used at 0.5% (Fleming and Young, 1940). It has been evaluated in concentrations of 1, 2, 3 g/L as selective agent for enumeration of *Lactobacillus casei* in fermented milk (Colombo *et al.,* 2014). It has been used in 0.2% for the enumeration of bifidobacteria in fermented dairy products(Lapierre *et al.,* 1992), and 15g/L is used as selective agent in a selective medium for *Listeria monocytogenes* isolation (Park *et al.,* 2014).

- Potasium tellurite:

Used for Baird-Parker culture medium. Inhibits enteric cocci and Gram-negative bacilli used at 0.1% (Fleming and Young, 1940). Zadik and coworkers (1993) use 0.5 mg/L for

verocytotoxigenic Escherichia coli O157 isolation. Zylber and Jordan (1982) use 2.5 mg/L for selective detection and enumeration of Actinomyces viscosus and Actinomyces naeslundii in dental plaque and Tanzer and coworkers (1984) use 1 mg/L for enumeration of *Streptococcus mutans* from the same source.

- Phenylethyl alcohol

Is a selective compound, it inhibits DNA synthesis and breaks the membranes lipids (Silver and Wendt, 1967) of the vast majority of Gram-negative bacteria (Berrah and Konetzka, 1962) at 0.25% (Lilley and Brewer, 1953), and performs anti-fungic activity (Koike *et al.,* 2010). It has been used to get Gram-positive bacteria from sheep rumen fluid (0.5% vol/vol) (Koike *et al.,* 2010). Sporulation inhibition also has been reported in *Neurospora crassa* (Lester, 1965), in *Bacillus megaterium* (Slepecky, 1963), in *Bacillus cereus* (Remsen *et al.,* 1966) and in *B. subtilis* (Richardson *et al.,* 1969). Clostridium grows poorly (Ninomiya *et al.,* 1970).

- Polymyxin B

Polymyxin B anchors to the cell membrane increasing permeability of Gram-negative bacteria (except *Proteus*). As a selective agent, it can be tested between 15 and 20 mg/L in culture media (Atlas, 2010). Among 10 - 30 µg/mL can be used in in combination with trimethoprim and cycloserine for selective *isolation of Clostrydium butyricum,* from human feces (Popoff, 1984).

- Nalidixic acid:

It inhibits partially *S. aureus* growth and totally Gram-negative bacteria growth truncating DNA synthesis. Furthermore, it is present in the NS (Non Spore Anaerobic) supplement used to favor non-spore forming bacteria growth (Atlas, 2010). 10 mg/L of this agent is used in the Columbia CNA Agar. In this culture medium grows yeasts, *Staphylococci, Streptococci,* and *Enterococci.* Some Gram-negative bacteria such as *Gardnerella vaginalis* and some *Bacteriodes sp.*, also grows (Atlas, 2010). 10, 15, 20 mg/L were evaluated as selective agent for enumeration of *Lactobacillus casei* in fermented milk (Colombo *et al.,* 2014).

- Gentamicin:

Inhibits the most of Gram-negative bacteria and *Staphylococcus* growth by truncating proteins synthesis. Streptococcus, *Clostridium* and *Bacterioides* could be resistant. Kneifel and Leonhardt (1992) propose to use1-4 mg/L for inhibition of bacteria from plant tissue. Merz and coworkers (1976) propose to use 50-100 µg/mL for inhibition of bacteria for isolation of fungi. Baker and coworkers (1973) established 5-10 µg/mL inhibits partially group B streptococci. Converse and Dillon (1977) propose to use 5 µg/mL for pneumococci isolation and Martin and coworkers (1972) evaluated 0.1 – 25 µg/mL in antimicrobial susceptibility of anaerobic bacteria isolated from clinical specimens.

- Streptomycin

Gardner (1966) established to use 500 µg/mL for selective isolation of *Microbacterium thermosphactum*, a Gram-positive bacterium. Yim and coworkers (2010) stablished to use 100 µg/mL to isolate *Sphingomonas sp.,* a strict anaerobic Gram-negative bacterium, from environmental samples. According to previous work in *R. albus*, Champion and coworkers (1988) showed that *R. albus* strains 7, 8 and B199 among others were resistant to 10-200 µg/mL streptomycin doing sensidisk agar diffusion test. In contrast, Tarakanov and Lavlinskii (2002) in their evaluation of the susceptibility 6 natural isolates of *R. albus* to several antibiotics found that just one growth in both 10 and 50 µg/mL concentration and other just in 10 µg/mL.

- Colisitn sulfate (polymyxin E)

It inhibits Gram-negative bacilli increasing permeability of the cell membrane (Atlas, 2010). Park and coworkers (2014) propose to use 10 mg/L for isolation of *Listeria monocytogenes.* Nordmannand coworkers (2016) found that 3.5 µg/mL is the optimal concentration to detect natural or resistant bacteria. Ehrmann and coworkers (2013) propose to use 3.75 mg/L for isolation of Capnocytophaga species from oral polymicrobial samples. Thayer and Martin (1966) established to use 20 µg/mL for isolation of *N. gonorrhoeae and N. meningitides*. Colisitin microdifusion from disk to agar is poor (Lo-Ten-Foe *et al.,* 2007), for this reason, evaluation in liquid medium is desirable.

- Amikacin

For example, 0.05 g/L is used in Wilkins-Chalgren Agar with Amikacin and 7% Sheep Blood BD™ which is a selective medium for the isolation of strictly anaerobic bacteria from clinical specimens (Wilkins and Chalgren, 1976), due to the amikacin, most facultative organisms are inhibited. Kneifel and Leonhardt (1992) evaluated among 4 – 16 mg/L () for isolation of bacteria from plant tissue. 0.075 g/L is included in the Bacteroides Bile Esculin Agar with Amikacin BD™.

- Trimethoprim

16 µg/mL is used for isolation of *Clostridium butyricum* from human feces (Popoff, 1984). 1.5 mg/L is used for isolation of Capnocytophaga species from oral polymicrobial samples (Ehrmann *et al.,* 2013). 20 mg/L is used for the isolation of *Arcobacter* from meats (de Boer *et al.,* 1996). And BBL™.SXT Blood Agar BD™ used for isolation and presumptive identification of group A and B streptococci contains 1.25 mg/L.

- D-cycloserine

10-20 g/mL is used in a selective culture for *Clostrydium butyricum, isolation from human* feces (Popoff, 1984). 250 µg/mL is used for detection of Clostridium difficile (Tait *et al.,* 2014). 0.1 g/L was used for isolation of *Methanosarcina sp.* (Zinder and Mah, 1979). 500 mg/L (cycloserine) cycloserine-cefoxitin fructose agar (CCFA) is used for *Clostrydium difficile* isolation (Fedorko and Williams, 1997).

2. Materials and Methods

2.1 Microorganisms

For this work, several strains of *Ruminococcus albus* from different collections and providers were used. *R. albus* strain 7 (DSMZ 20455) was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ) culture collection. The *R. albus* strains 7, 8, SY3 and B199 used in the step of evaluation of the chemical-defined medium were obtained from the culture collection of the Department of Animal Sciences of the University of Illinois at Urbana-Champaign (Urbana Illinois – USA). Strain 7 was reported by Bryant and coworkers (1958). Strain 8 was isolated by Hungate and Stack (1982). Strain SY3 came from the culture collection of the Research Institute Aberdeen, Scotland. This strain was originally isolated from a sheep's rumen (Wood *et al.,* 1982). And strain B199 was obtained from Terry Miller (New York State Health Department, Albany) (Russell, 1985).

Inoculations and other handling procedures of anaerobic grown cultures of this work were carried out by injection with disposable syringes according to Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ) recommendations (available in: https://www.dsmz.de/). First, sterilizing the butyl rubber stopper by flaming it using a drop of ethanol which has been placed previously on the top. Overpressure in the bottle or tube due to microbial growth (e.g., gas production by fermentation) was released removing the gas excess by puncturing the rubber stopper with a sterile injection needle allowing gas fills the syringe and then it was withdrawed. Then, the vial was turned with the culture upside down and the syringe was filled with the needed amount of liquid. Finally, needle and filled syringe was withdrawed carefully and transferred to a fresh medium.

The first *R. albus* strain 7 (DSMZ 20455) was reactivated according to DSMZ recommendations (Appendix A) but omitting gasification with oxygen-free gas and suspending it in 0.5 mL liquid medium as fast as possible given to lab conditions of the Instituto de Biotecnología de la Universidad Nacional – IBUN (Bogotá, Colombia). Then it was injected in four 50 mL of culture medium serum bottles. Two of them were incubated at 37 °C and the other two at 30 °C. The second acquired strain 7 of *R. albus* (DSMZ-

20455) was reactivated according to DSMZ recommendations but in this occasion, procedure was carried out under oxygen-free CO_2 gas stream according to USBA (Bogotá, Colombia). Then it was injected in 10 ml fresh culture medium tubes which were incubated at 37°C.

Strains 7, 8, SY3 and B199 were stored at temperatures below -130°C liquid nitrogen tank in 8 mL serum bottles with agar slant prepared under CO_2 oxygen-free gas, inoculated with the respective strains and incubated till late exponential phase. Stored serum bottles with cultures of strains were warmed to 37 °C before culture transferring. Then at least of 500 µL of liquid of each serum bottle were taken with a syringe and transferred to tubes with fresh medium prepared as described above. Tubes were incubated at 37°C till turbidity was observed (from 24 to 48 hours).

2.2 Culture media composition

2.2.1 RGC-based culture medium

The culture medium used was based on RGC (Rumen Fluid Glucose Cellobiose) medium (Makkar and McSweeney, 2005) composition as follows: 1L of culture medium contains 5.0 g yeast extract, 2.0 g of cellobiose, 300 mL of mineral solution I, 300 mL of mineral solution II, 2mL of 0.1% resazurin solution, 12.0 g $NaHCO_3$, 0.8 mL fatty acid mixture 2g Cysteine-HCl*$2H_2O$. The final volume was completed with 400 mL of distilled water. The mineral solution I composition was: 0.3% K_2HPO_4; the mineral solution II was: 0.3% KH_2PO_4, 0.6% $(NH_4)_2SO_4$, 0.6 % NaCl, 0.06% $MgSO_4$*$7H_2O$, 0.06% $CaCl_2$*$7H_2O$. The fatty acids mixture solution contains: 10.0 mL Isobutyric acid, 10.0 mL isovaleric acid, 10.0mL 2-Methylbutyric acid and 70 mL of distilled water.

2.2.2 DSMZ436 culture medium

DSMZ436 culture medium was prepared using the reagents and composition recommended by the DSMZ. 1L of culture medium contains 5.0 g tryptone, 2.0 g yeast extract, 3.0 g glucose, 2.0 g cellobiose, 40 mL of mineral solution I, 40 mL of mineral

solution II, 1 mg resazurin, 4.0 g Na_2CO_3, 1.0 mL fatty acid mixture 500 mg Cysteine-HCl*H_2O. The final volume was completed with 920 mL of distilled water. The mineral solution I composition is: 0.6% K_2HPO_4; the mineral solution II one is: 0.6% KH_2PO_4, 2.0% $(NH_4)_2SO_4$, 1.2% NaCl, 0.25% $MgSO_4$ *7H_2O, 0.16% $CaCl_2$*7H_2O. The fatty acids mixture contents: 10.0mL Isobutyric acid, 10.0 mL isovaleric acid, 10.0mL 2-Methylbutyric acid and 70mL of distilled water.

2.2.3 Chemical-defined medium

1 L of Chemical defined culture medium (Makkar and McSweeney, 2005) has the following composition: 40 mL of mineral solution I, 40 mL of the mineral solution II, 1 mL of 0.1% resazurin solution, 0.5 mL of 0.1% hemin solution, 1 mL of Pfemming's solution, 4 g of $(NH_4)_2SO_4$, 860 mL of distilled water, 10 mL of VFA (volatile fatty acids) solution, 10 mL of vitamin B solution, 4 g of cellobiose or cellulose (depending on the of the carbon source of the culture medium) and 4 g of $NaHCO_3$.

The mineral solution I composition is: 0.6% K_2HPO_4. The mineral solution II one is: 0.6% KH_2PO_4, 1.2% NaCl, 0.12 % $MgSO_4$ * 7H_2O, 0.12 % $CaCl_2$*7H_2O. 100 mL of hemin solution contains 100 mg of hemin and 1 mL NaOH 1N. 1 L of the Pfemming's solution contains: 0.5 g EDTA, (ethylenediaminetetraacetic acid), 0.1g $ZnSO_4$, 7H_2O, 0.03 g $MnCl_2$*4H_2O, 0.03 g H_3BO_3, 0.2 g $CoCl_2$.6H_2O, 0.01 g $CuCl_2$.2H_2O, 1.5 g $FeCl_2$.4H_2O, 0.02 g $NiCl_2$.6H_2O, 0.03 g Na_2MoO_4.2H_2O, 0.01 g Na_2SeO_3. The fatty acids mixture contents: 700mL NaOH 0.2N, 13.7 mL acetic acid, propionic acid 6 mL 3.68 mL butyric acid, 1.1 mL isobutyric acid, 0.94 mL 2-methylbutiric acid, 1.1 mL n-valeric acid, 1.1 mL isovaleric acid, 340 mg PAA (phenylacetic acid) and 376 mg PPA (3-phenylpropionic acid), pH is adjusted to 7.5 and the volume to 1 L of Schaefer vitamin B solution contains: 20 mg thiamine HCl, 20 mg Ca-D-pantothenate, 20 mg nicotinamide, 20 mg riboflavin, 20 mg pyridoxine-HCl, 1 mg para-aminobenzoic acid, 0.5 mg biotin, 0.125 mg folic acid, 0.2 mg vitamin B12, 0.125 mg tetrahydrofolic acid. And 200 mL of cysteine sulfide solution contains: 1.25 g NaOH, 5 g Cysteine-HCl, and 5 g Na_2S*9H_2O.

2.3 Isolation and identification of *Ruminococcus albus* from rumen fluid

This first stage was carried out in CORPOICA (Mosquera, Colombia) in 2012. The objective was obtaining isolates of *R. albus* from rumen fluid of individuals corresponding to Colombian beers of cattle in order to evaluate their hydrogen production on an agro-industrial vegetal matter.

2.3.1 Animals

A sample of 50 to 100 mL of both liquid and solid phase of rumen fluid was taken using an esophageal catheter of adult alfalfa-fed individuals belonging to the following Colombian bovine breeds: Blanco orejinegro (white with black ears), Costeño con cuernos (hormed-coastal), Hartón del Valle (from the Colombian department “Valle del Cauca”), Romosinuano (blunt from Sinu river), Sanmartinero (From San Martin, an eastern town of Colombia).

2.3.2 Roll-tube technique for isolation of cellulolytic bacteria

For culture medium preparation liquid components were mixed first, then the nitrogen sources and the carbon source were added. Subsequently the pH was adjusted to 7.2, the sodium carbonate was added and the medium was pre-reduced boiling for 15 minutes. Later it was cooling down under oxygen-free CO_2 gas stream, cysteine-HCl as reducing agent was added and when the color of the medium torns into transparent it was placed into tubes or serum bottles gasified with CO_2. Culture medium was autoclaved under 121°C and 20 psi. for 15 minutes.

The roll-tube technique developed by Hungate (1950) was used for isolation of *R. albus* which has been successful for isolation of this bacterium among other oxygen-sensible microorganisms (Hungate, 1950; Hungate, 1976). Tubes and/or serum bottles with rubber stoppers and aluminum seals are used to keep the anaerobic environment in both gas and liquid phase of the tube or bottle. To get pure cellulolytic isolations from rumen fluid, the previously mentioned medium was prepared as described before, with addition of 1.5%

agar. As carbon source, 1% pebble milled cellulose (PMC) (Whatman N°1 filter paper) and 0.05% cellobiose were added to one set of tubes, while the other set contained 1% carboxymethyl-cellulose (CMC), and cellobiose at 0.05% as well. Dilution medium was also prepared as previously described but the only compounds it contained were mineral solutions I and II, the resazurin, the $NaHCO_3$ and the cysteine-HCl. This medium were placed in tubes; 9 mL per tube of dilution medium, and 4.5 mL per tube of the PMC or CMC media.

Then serial dilutions were prepared transferring 1mL of fresh rumen fluid to a 9 mL dilution medium-containing tube and so on 9 times, till achieve 10^{-9} dilution. Immediately both CMC, and PMC 4.5 mL media tubes were inoculated with 0.5mL of the 10^{-8} y 10^{-9} dilution tubes by triplicate. Both CMC, and PMC media tubes should be previously melted and kept at 45 °C in the moment of inoculation. After inoculation, tubes were placed horizontally on a roll-tube machine which turns around allowing agar to adhere to the tube wall forming a tinny agar film. Rolled tubes were incubated at 39 °C for 3 to 8 days till single colonies with cellulolytic halos were observed.

To recover single isolations, tubes were opened and gasified with oxygen-free CO_2. Carefully the colony was punctured with a curved-tip Pasteur pipet connected to a silicon CO_2 gas pipeline (CO_2 should pass through a 0.2 m pore to avoid microbial contamination in the process). Then colony was transferred to a tube containing the previous described liquid medium with 0.2% cellobiose as carbon source. Inoculated tubes were incubated from 24 to 48h at 39 °C till turbidity was observed.

2.3.1 Morphological identification of microorganisms

After turbidity was observed in liquid culture media tubes, a drop of culture was placed on a slide to observe microscopic morphology under 100x phase contrast microscope. To confirm morphology, Gram staining was carried out to such isolations where cocci were observed. Then isolations were stored at 4 °C, afterwards isolations were re-inoculated for cryopreservation.

2.3.2 Cryopreservation procedure

Preservation of isolated colonies was carried out in 2 mL cryo-tubes adding 800 µL of exponential-phase growing culture medium and 200 µL of oxygen-free glycerol under oxygen-free CO_2 gas stream.

2.3.3 Molecular identification of presumptive isolations

Such colonies which morphology corresponded to *R. albus* microscopic description: single and paired gram positive cocci (Hungate, 1957) were identified by amplification and sequencing of the 16S rDNA carried out by the Laboratoire d'Electrochimie et de Physicochimie des Matériaux et des Interfaces - LEPMI (Grenoble, France).

2.4 Evaluation of DSMZ 436 culture medium

The first growth evaluation in DSMZ 436 was carried out in the Instituto de Biotecnologia de la Universidad Nacional – IBUN (Bogota, Colombia), and was prepared as following: All medium components were mixed and pH was adjusted to 5.8. Resazurin was added and the medium was boiled till it turns from pink to transparent, which means oxygen has been depleted. Culture medium was placed in bottles into the anaerobic chamber under 80% N_2, 10% CO_2 and 10% H_2 Oxygen-free atmosphere. The bottles with medium were sterilized under 121 °C and 15 psi. for 20 minutes. Resuspension of lyophilized cells were inoculated in four bottles; two were incubated at 30 °C and the other two at 37 °C for nine days, till turbidity was observed. When culture medium exhibit growth, 0.5 mL of the inoculum was transferred to a fresh 50 mL medium and was incubated at 37 °C for Gram staining. Afterwards working culture was preserved at 4 °C.

This medium was evaluated for a second time in the Unidad de Saneamiento y Biotecnologia Ambiental – USBA at the University Pontificia Universidad Javeriana in Bogotá, Colombia. pH adjustment was done before the boiling pre-reduction (as the previous method), the pre-reduction was done under N_2 gas and cooling down under CO_2. Culture medium was autoclaved at 121 °C and 15 psi. for 20 minutes. The reviving inoculum

was placed with syringes in two 10 mL tubes which were incubated for nine days till turbidity was observed. When culture medium exhibit growth, 0.5 mL inoculum was transferred to a fresh 50 mL medium with addition of 50 µL of 1 mg/mL hemin solution and then, Gram staining was performed. Afterwards working culture were preserved at 4 °C.

2.5 Evaluation of chemical- defined culture medium

This evaluation was carried out in The Animal Sciences Laboratory at the University of Illinois at Urbana – Champaign (Illinois, United States of America).

Four strains of *R. albus:* 7, 8, SY3 and B199 were reactivated in 10 mL tubes of a chemical defined culture medium which was prepared as following: 1 L of the culture medium has 40 mL of mineral solution I, 40 mL of mineral solution II, 1 mL of 0.1% resazurin solution, 0.5 mL of 0.1% hemin solution, 1 mL of Pfemming's solution, 4 g of $(NH_4)_2SO_4$, add 860 mL of distilled water, boiled in microwave and cooled down under oxygen-free CO_2 gas. When medium was at environment temperature, 10 mL of VFA (volatile fatty acids) solution were added, 10 mL of vitamin B solution, 4 g of cellobiose or cellulose (depending of the aim of the medium) and 4g of $NaHCO_3$. Then the pH was adjusted to 6.8 and the medium was transferred to an anaerobic chamber under 100% CO_2 gas, 20 mL of cysteine-sulfide solution were added, when the pink color was turned into transparent, it was placed into rubber-stopper tubes or bottles and autoclave at 121 °C and 15 psi for 20 minutes.

2.5.1 Cryopreservation procedure

For cryopreservations, chemical-defined medium tubes were prepared, autoclaved and inoculated with each one of the *R. albus* strains: 7, 8, SY3 and B199. Tubes were incubated at 37 °C for 24 hours. 5 ml bottles of chemical defined medium with 1.5% agar addition were prepared and autoclaved. Medium was keep at 45 °C in such way that agar remained melted. Bottles were inoculated with 200 µL of the 24 hours incubated liquid medium with each strain. Bottles were homogenated and incubated in inclined position at 37°C to form agar slants, for 10 hours or till cultures reach late exponential growth phase. Immediately, agar slants bottles were stored at temperatures below -130 °C liquid nitrogen tank or in -80 °C freezer.

2.5.2 Molecular identification of presumptive strains

In order to ensure purity of cultures, chemical defined medium was prepared with 1.5% of agar addition and placed in plates in anaerobic chamber. 0.1ml of 24 hours incubated culture with each strain were placed on the agar surface of one plate per strain and streaking was carried out to get single colonies. Plates were incubated for 24-48 hours till single colonies were observed. Single colonies of each one of the strains were placed in chemical defined liquid medium tubes and incubated for 24 hours. Gram staining were carried out and 100x microscopic morphology observation was done.

For confirmation of identity, 24 hours grown cultures in liquid medium of each strains were transferred to 15 mL falcon tubes and centrifuged at 10000 rpm. for 10 minutes at 4 °C. Supernatant was removed and DNA extraction was carried out using a modified PowerSoil® kit protocol (Appendix B). Extracted genomic DNA was checked by electrophoresis in a 1% agarose gel stained with bromide ethidium, runt for 40 minutes at 100 volts. Later 16S rRNA subunit gene was amplified using universal primers 27_F and 1492_R. For a total reaction volume of 25 µL in a 200 µL PCR tube, reagents volumes were added as follows: 2.5 µL of 10 µM 27_F primer, 2.5 µL of 10 µM 1492_R primer, 2 µL previously extracted genomic DNA, 12 µL of MasterMix and 6 µL of molecular grade water. PCR tubes were placed in a thermocycler which was programmed as follows: 10 minutes for 94 °C initial DNA denaturing step, 34 repetitions of 94 °C of denaturation step for 30 seconds, 53 °C for 30 seconds and 72 °C for primer hybridization and elongation steps, and a final elongation of 72 °C for 7 minutes. Further PCR products were checked in a 1% agarose gel electrophoresis run at 100 volts for 40 minutes. PCR products were purified with the QIAquick® PCR purification kit (Sample and Assay Technologies) as described in Appendix C, and sent for sequencing in both directions (ACGT, Inc., Wheeling, IL).

2.5.3 Growth curve of R. albus strains 7, 8, SY3 and B199 in the chemical defined medium

To determine growth curve *R. albus* strains 7, 8, SY3 and B199, chemical defined medium 10 mL tubes were prepared. Each strain was inoculated in three tubes which were inoculated at 37 °C and in other three tubes which were inoculated at 39 °C, to have a total of

six tubes per strain. To stablish de blank, the OD (optical density) of one non-inoculated fresh medium containing tube was read in a Spectromic 20D spectrophotometer at 600 nm and adjusted to 0. Then, the OD of the other tubes before being inoculated was read. When tubes were inoculated, again the OD. This registered value belonged to the time 0 reading. The OD of whole tubes was measured once per hour during 14 hours. Then for strains 7, 8 and SY3, a last OD measurement at 24 hours after inoculation was taken.

2.5.4 Evaluation of agents to improve culture medium selectivity

According to literature revision and laboratory availability, *R. albus* resistance to several selective agents were evaluated to select such ones which would serve as selective components in a culture medium for *R. albus* isolation. The following agents were evaluated in liquid chemical-defined medium 10 mL tubes by triplicate, adding the agent of interest. Growth was checked by observing turbidity after 24 hours and 48 hours of incubation.

To investigate if the four strains of *R. albus* 7, 8 SY3 and B199 grow at low pH, they were inoculated in tubes which pH was previously adjusted to 6.0, and incubated for 24 hours at 37 °C. The four *R. albus* strains growth was also evaluated at 0.5% propionic acid, in 5% NaCl, 00.5% of Lithium chloride (LiCl),

Antibiotics resistance also was evaluated in chemical-defined liquid medium because is more confident than using agar-diffusion methods. For example, Huys and coworkers (2002) demonstrated that major errors can occur in determining gentamicin resistance using sensidiscs tests because of antibiotic diffusion in the agar. First, 10 mL of chemical-defined liquid medium tubes were prepared and autoclaved. Stock solutions of the following antibiotics were prepared, sterilized by filtration and added to each medium tube. Then tubes were inoculated with 24 hours grown cultures of each strain and incubated for 24 hours at 37 °C. Polymyxin B resistance of the four *R. albus* strains was evaluated at 15 µg/mL, nalidixic acid at 25 µg/mL, gentamicin at 5 µg/mL, and streptomycin at 50 µg/Ml.

3. Results

3.1 Isolation and identification of *Ruminococcus albus* from rumen fluid of individuals belonging to five bovine Colombian breeds

After one week of incubation, growth in roll tubes were checked and visible colonies were recovered in cellobiose liquid medium under oxygen-free CO_2. A total of 161 isolations were transferred and incubated at 39 °C for 24-48 hours or till growth was observed: 62 (39%) were recovered from CMC medium and 99 (61%) from PMC (Figure 3 a). From Blanco orejinegro individual, 20 colonies were recovered: 7 from CMC medium and 13 from PMC medium. From Costeño con cuernos individual, 4 colonies were recovered: 2 from CMC medium and 2 from PMC medium. From Harton del Valle individual, 42 colonies were recovered: 15 from CMC medium and 27 from PMC medium. From Romo-sinuano individual, 33 colonies were recovered: 13 form CMC medium and 20 from PMC medium. And from Sanmartinero individual, 62 colonies were recovered: 25 form CMC medium and 37 from PMC medium.

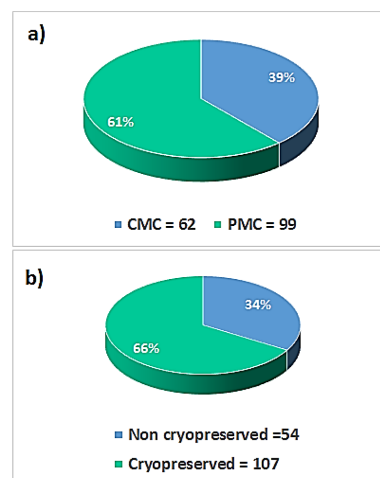


Figure 3: 161 isolations were obtained from rumen fluid of five Colombian bovine breeds. a) Percentage of isolations recovered using CMC and PMC as carbon source. b) Percentage of isolations able to be cryopreserved after storage at 4°C, reinoculated and incubated at 39°C.

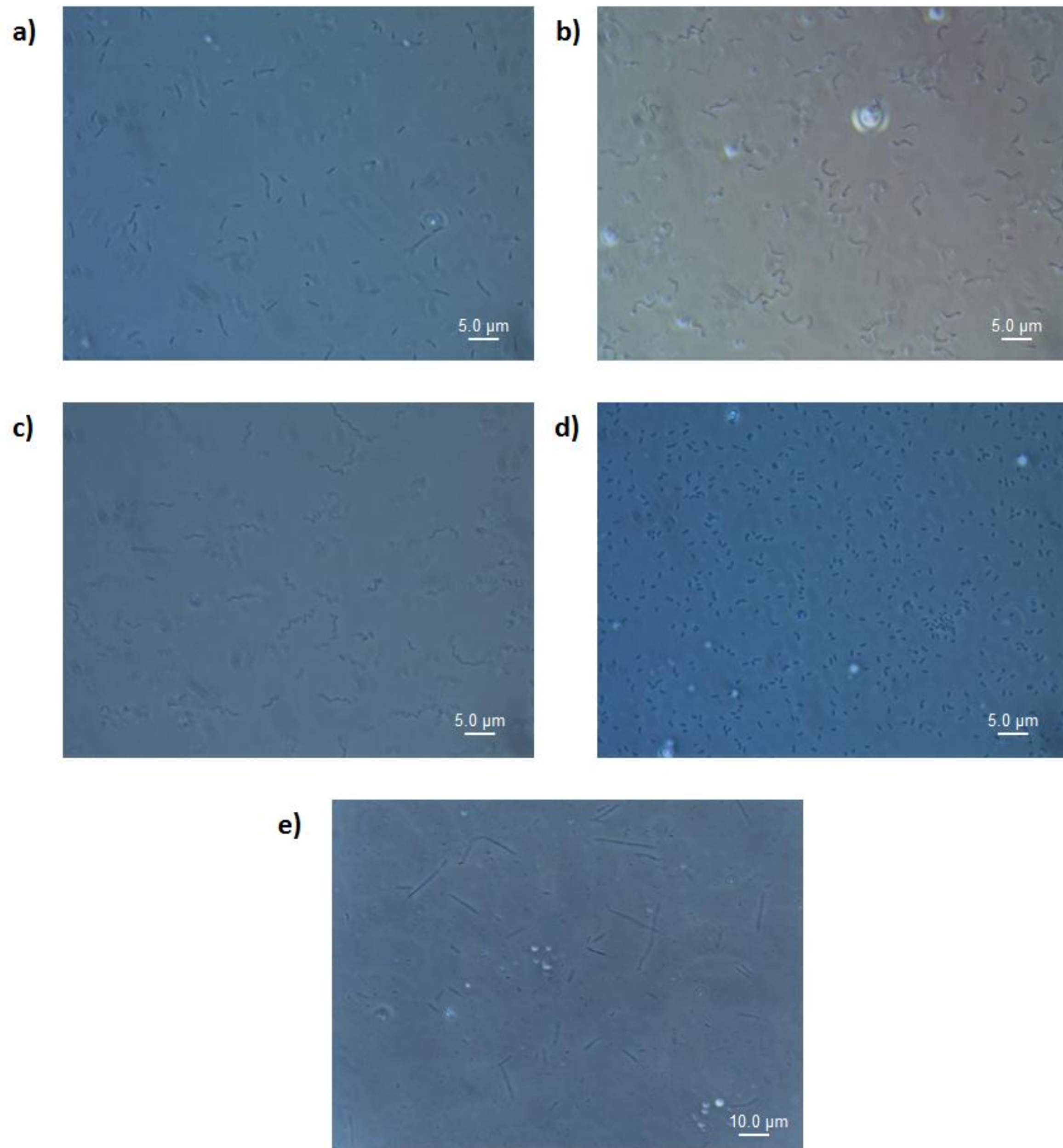


Figure 4. Morphologies of isolations obtained from rumen fluid using PMC and CMC independently as carbon source in a RGC-based culture medium. a) Vibrio shape rods of an isolation obtained from Harton del Valle individual in PMC medium. b) 'S' shape filaments of an isolate obtained from Romo sinuano individual in PMC medium. c) Spiral shape cells of an isolate obtained from Sanmartinero individual in CMC medium. d) Cocci obtained from Blanco orejinegro individual in CMC medium. e) And rods in chains obtained from Sanmartinero individual in PMC medium.

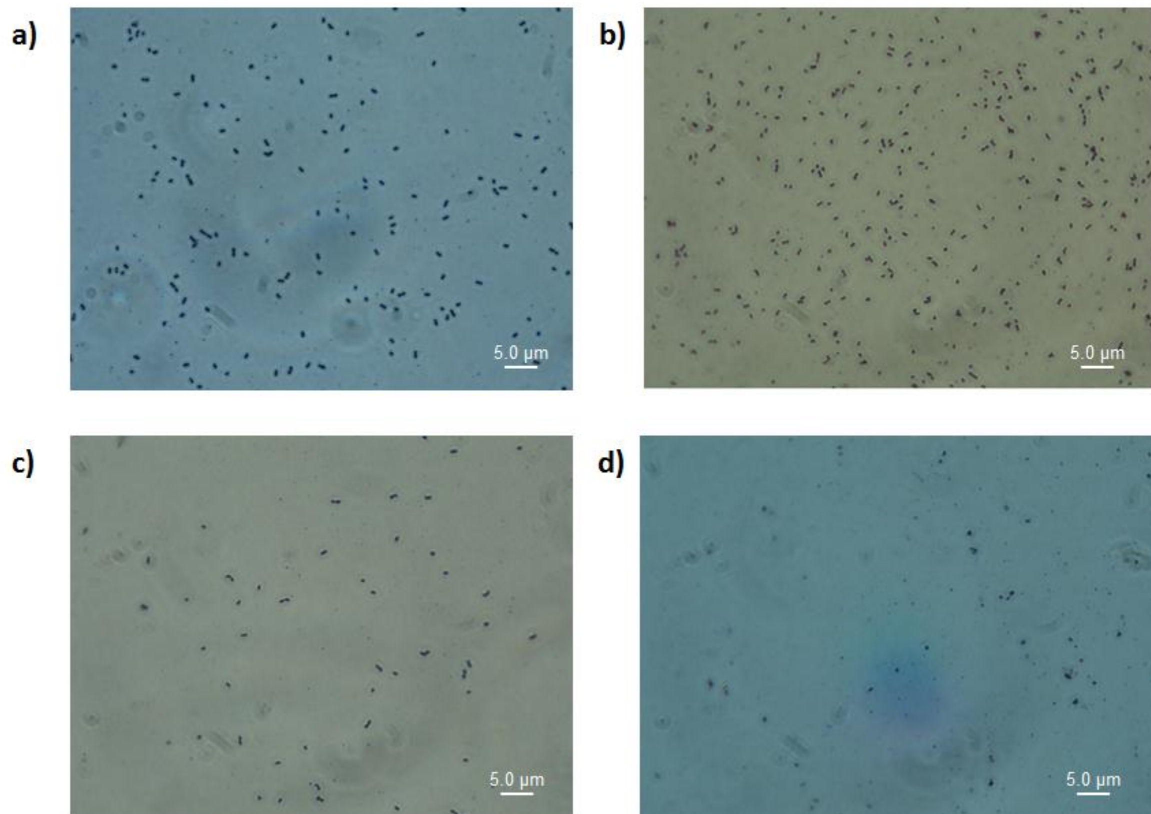


Figure 5. Morphology and Gram staining of cocci shape pure cultures recovered after cryopreservation. a) Isolate obtained from Blanco orejinegro individual in CMC medium. b) Isolate obtained from Romo sinuano individual in CMC medium. c) Isolate obtained from Romo sinuano individual in CMC medium. d) Isolate obtained from Costeño con cuernos individual in PMC medium.

Once isolations grown, phase contrast microscopic was used to check the morphology. 72 of the 161 isolations had a homogeneous morphology, which means that isolations were pure, while more than one morphology was observed in the remaining 89 isolations. The most observed shapes of isolations were vibrio-like, rods, “S” shape filaments, rods in chains, spirals and cocci (Figure 4). All these shapes were observed in both PMC and CMC isolations. In 54 isolations, cocci shape of cells was observed but just 12 of them were pure. Gram staining was carried out on all 54 isolations. Concerning to 12 pure ones, just two had Gram-negative cocci, the remaining nine were Gram-positive.

All the 161 colonies were stored at the fridge and revived for cryopreservation, but just 107 grew again and could be cryopreserved (Figure 3 b). To establish the identity of the nine cocci cultures, cryopreserved tubes were inoculated in fresh cellobiose medium and

incubated for 3 days. Just four of the nine cultures grown (Figure 5) and the 16S rDNA ribosomal subunit gene could be amplified and sequenced. Amplification, sequencing and identification was carried out by the Laboratoire d'Electrochimie et de Physicochimie des Matériaux et des Interfaces - LEPMI (Grenoble, France). For three of the four isolations, sequencing results were consistent. The isolation corresponding to the figure 5.a) matched in a 100% with the *Streptococcus equinus* strain C3, the isolation corresponding to the figure 5.b) matched in a 100% with *Streptococcus equinus* strain W3, and the isolation corresponding to the figure 5 c) matched in a 100% with *Streptococcus bovis* strain JB1. Despite all three strains had *R. albus* morphology and grew in CMC and PMC medium, none of them were *Ruminococcus albus.* The isolate obtained from Costeño con cuernos individual in PMC medium (Figure 5 d), could not be identified maybe because actually was not pure.

3.2 Evaluation of DSMZ 436 culture medium

Once *R. albus* strain 7 obtained from DSMZ was inoculated in the DSMZ 436 culture medium, it took nine days of incubation for bottles at 30 °C to exhibit turbidity; in those at 37 °C growth was not observed. Then an inoculum of the fresh grown medium was incubated at 30 °C for 48 hours. A drop of this new grown culture was fixed in a glass slide and Gram staining was carried on, observing single and by pair Gram-positive cocci of several sizes, but Gram-positive rods also were observed (Figure 6 a). It means the culture was contaminated. To suppress contamination, serial dilutions of 1mL of this grown culture medium were inoculated in fresh growth medium till dilution 10^{-6} and incubated at 37 °C for 24-48 hours. Then Gram staining of grown dilution tubes was carried on. In slides belonging to the dilution10^{-1} and 10^{-2}, Gram-positive and Gram-negative rods were observed respectively (Figures 6 b and 6 c) and in the remaining dilution tubes several sizes of cocci were observed (Figure 6 d).

According to slides of the most of dilutions, culture seems to be pure. But to ensure it, it was made the decision to get isolations by streaking in solid DSMZ 436 medium adding 1.5% of agar and microcrystalline cellulose as carbon source instead of cellobiose. Agar was placed in plates as quick as possible to avoid oxygen introduction but despite of this, medium turned into pink and after 8 days of incubation at 37 °C, no growth was observed.

Meanwhile, culture medium bottles of dilutions which seems to be pure, were stored at 4 °C, but when fresh medium was inoculated with these cultures, no growth was observed.

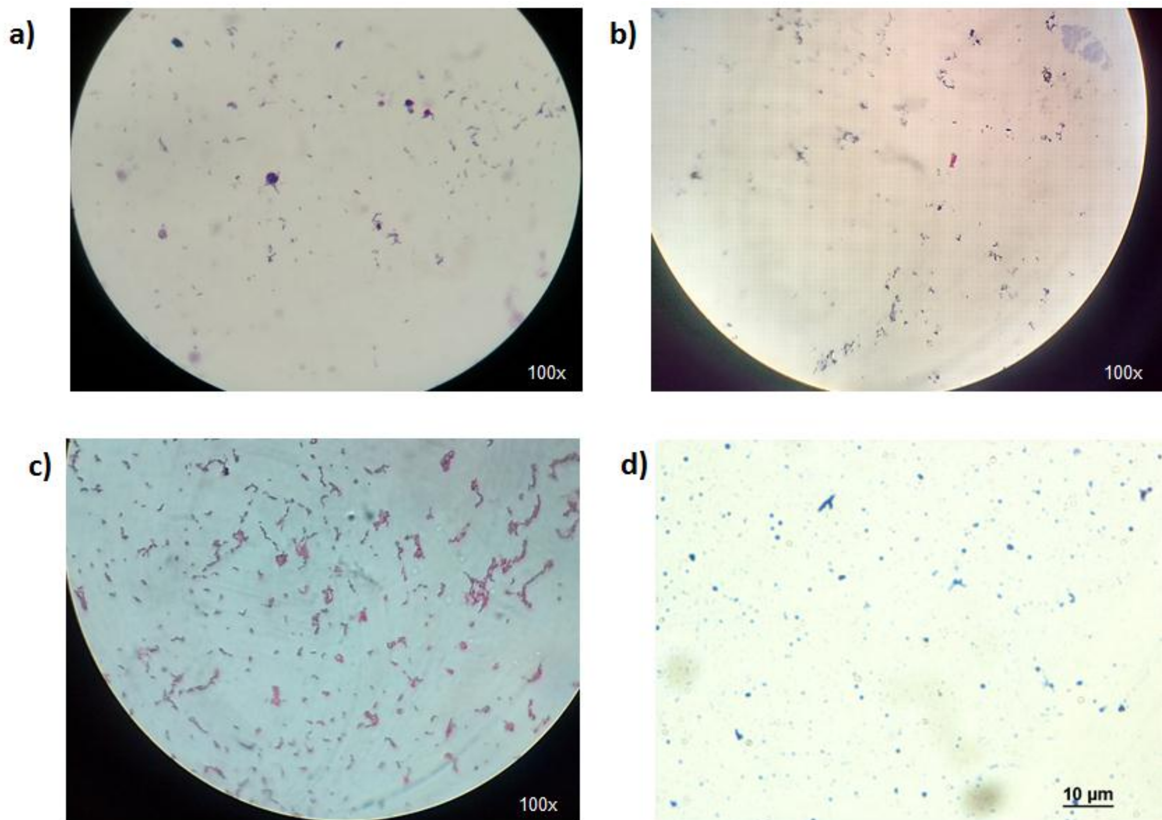


Figure 6. Morphology and Gram staining of *R. albus* strain 7 cultures in the first trait for culturing this strain. a) First Gram staining. Both rods and cocci are observed. b) Morphology and Gram staining of the dilution dilution10^{-1} c) Morphology and Gram staining of the dilution dilution 10^{-2}. Morphology and Gram staining of the dilution dilution10^{-5}.

Again DSMZ 436 culture medium was prepared and inoculated with resuspended lyophilized strain 7 of *R. albus*. When cultures were incubated at 37 °C, growth was observed nine days later. Microscopic observation of fresh drop of culture was done observing single, pairs of cocci and sporadically cocci arranged in clusters. When Gram staining was carried on, Gram-negative cocci and clusters and a Gram-negative filamentous structure was observed as well (Figure 7 a). Curiously when bottle was stirred manually, the cell growth emerged from the button of the bottle as a floc. An inoculum of the growth culture was placed in a fresh medium bottle adding 50µL of 0.1% hemin which was incubated at 37 °C for 24 hours. A new Gram staining was carried readjusting the

washing with alcohol-acetone step from 30 seconds to 15 seconds, and in this time Gram-positive cocci and cell clusters were observed (Figure 7 b). The culture was stored at 4 °C but when fresh medium was inoculated with this culture, growth was never observed after days of incubation.

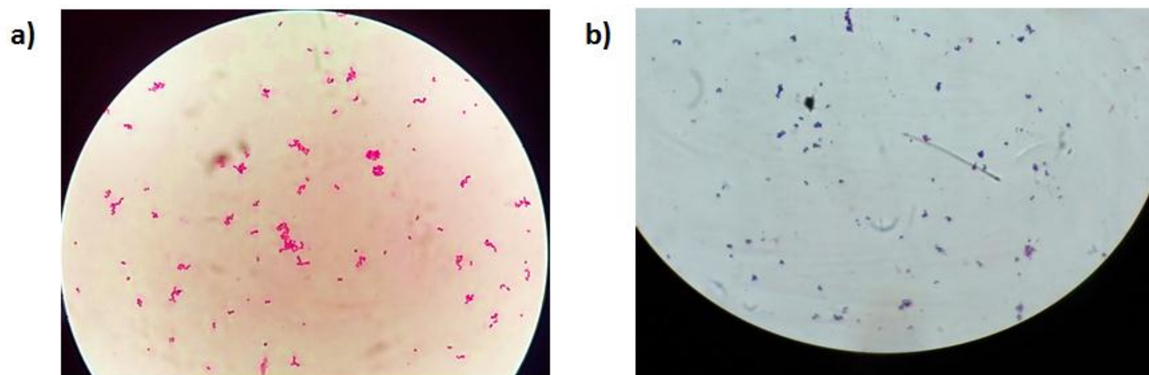


Figure 7. Morphology and Gram staining of *R. albus* strain 7 cultures in the second trait for culturing this strain. a) First Gram staining which seems Gram-negative b) Second Gram staining which seems Gram-positive.

3.3 Evaluation of chemical- defined culture medium

After 24 to 48 hours of incubation of reactivated strains 7, 8, SY3 and B199 of *R. albus* in chemical-defined medium, growth of four strains was observed. Microscopic observation and a new inoculum was carried on, observing Gram-positive single and pairs of cocci for strains 7, 8 and B199 (Figure 8: a, b, d), and for strain SY3 (Figure 8 c) single, pairs and clusters of cocci were observed, which was consistent with a floc formed in the bottom of the tube. Morphological uniformity was observed in all four strains. To secure purity, chemical defined medium agar plates were inoculated with the four strains in the anaerobic chamber by streaking. Plates were incubated anaerobically at 37 °C for 48 hours. Tubes were stored for 3 days at 4 °C but when a new inoculum of these cultures was intended to be incubated, just *R. albus* SY3 and B199 grown. A single colony of each strain was transferred from the agar plate to a liquid medium and incubated 24-48 hours at 37 °C observing growth of four strains. From this point of experiments ahead, strains were maintained at 37 °C all the time, renewing inoculations each two days, or once a week, even it was proved strains could revive from a 20-days old culture if it is maintained at this temperature.

3.3.1 Molecular identification of presumptive strains

16S rRNA ribosomal subunit gene sequence obtained for each of presumptive strain was compared by nucleotide BLAST to determine their similarity and identity (BLAST: Basic Local Alignment Search Tool, available in The National Center for Biotechnology Information - NCBI at http://www.ncbi.nlm.nih.gov/). All four strains matched in a 100% identity and a 100% of similarity to *R. albus* 7, 8, SY3 and B199, which means cultures were pure and were actually what presumptively they were.

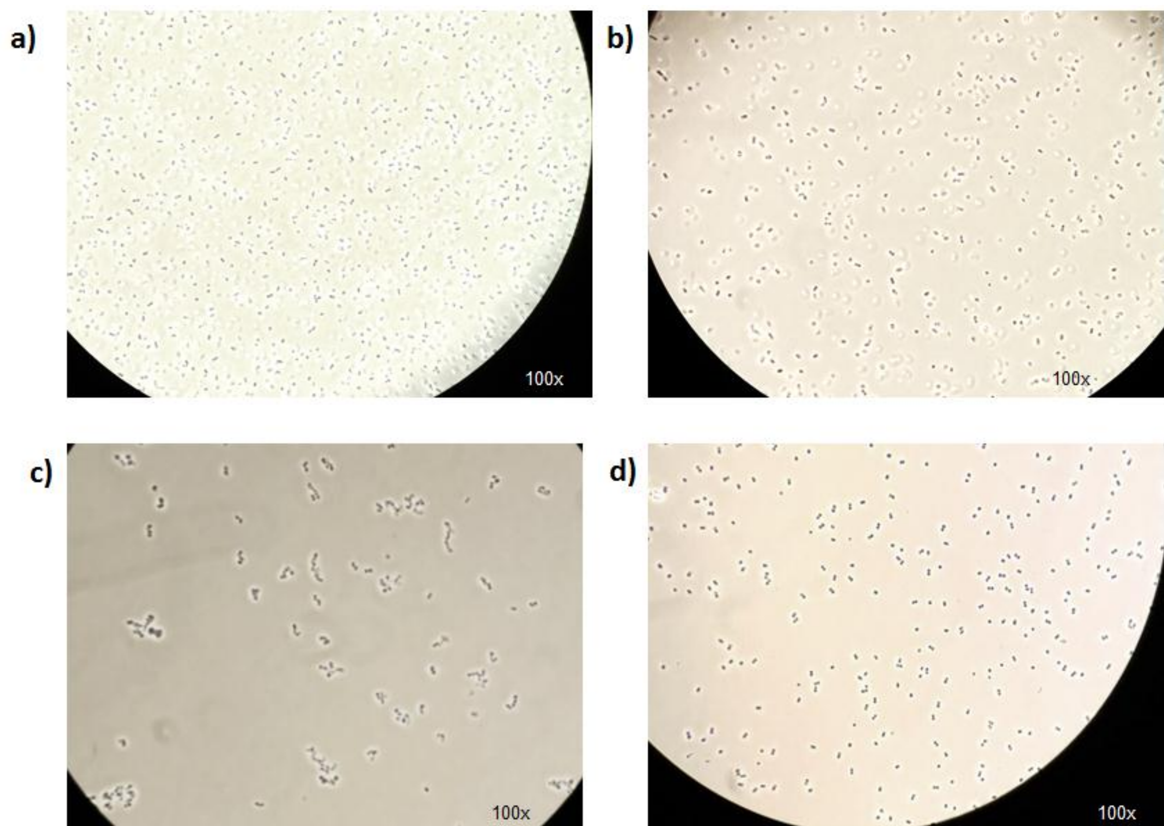


Figure 8. Morphology of *R. albus* strains at 24 hours of incubation at 37°C. a) strain 7, b) strain 8, c) strain SY3 and d) strain B199.

3.3.2 Growth curve of *R. albus* strains 7, 8, SY3 and B199 in the chemical defined medium

The four strains of *R. albus* exhibited similar growth curves among them (Figure 9); they reached late exponential phase at 8 – 10 hours of incubation both at 37 °C and 39 °C. The slope of this phase is slightly higher at 39 °C than at 37 °C, which is expected because is the natural temperature of the rumen, but at both temperatures the four strains can growth pretty well.

3.3.3 Evaluation of agents to improve culture medium selectivity

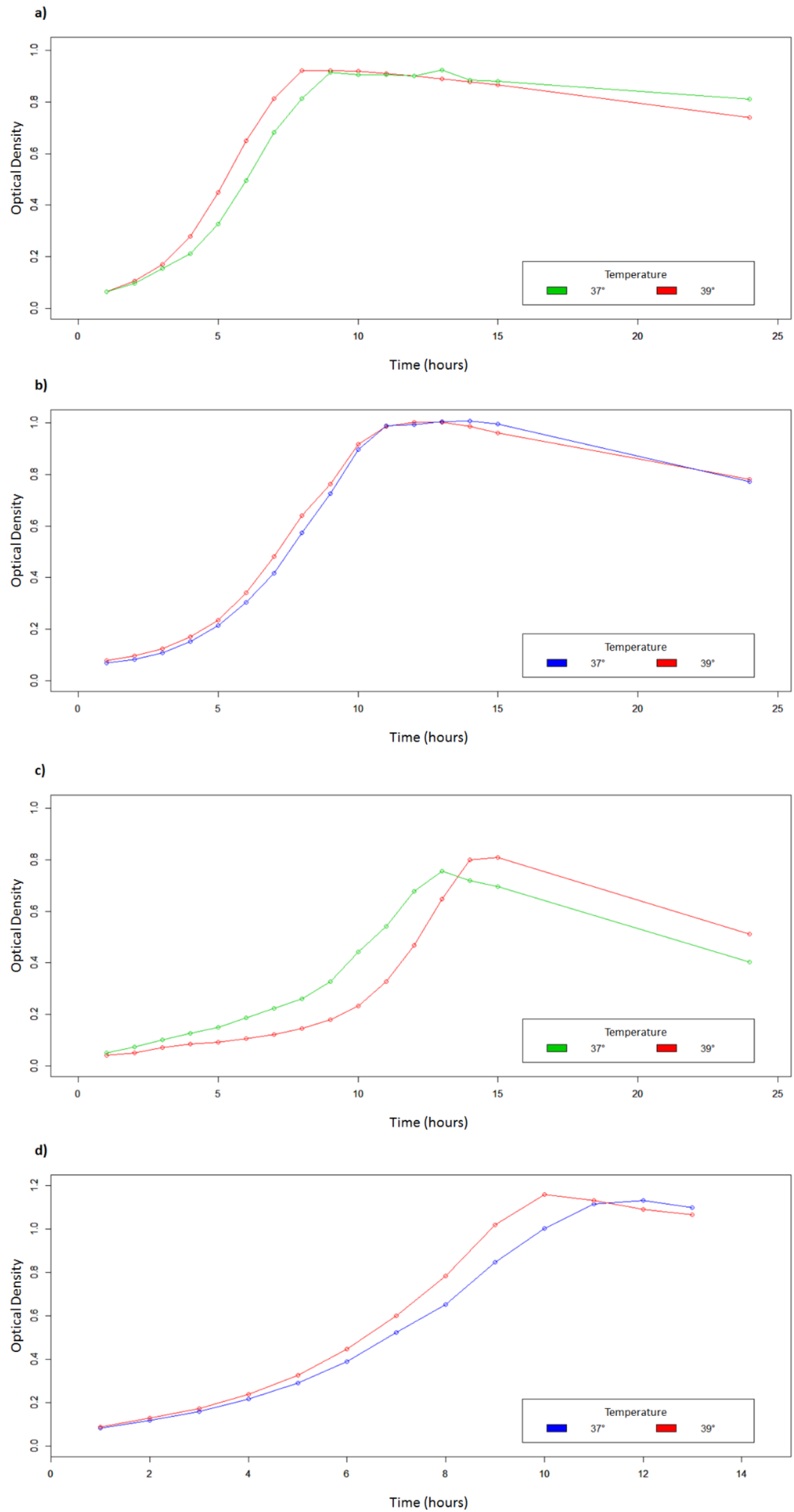
Almost all the strains grown under 0.5% LiCl and 0.5% propionic acid presence in the culture medium after 24 hours of incubation (Table 1). But it is necessary to remark that in the strain B199 tube, growth was observed after 48 hours of incubation, even the turbidity was less than observed when no selective agent was present. Three of the four strains grew under presence of 25 µg/mL nalidixic acid as well. No growth was observed in the strain 8 even at 48 hours of inoculation. None of the four strains grew under 5% NaCl nor 15 µg/mL polymixin B. Just strain 7 exhibit resistance against 5 µg/mL gentamycin and 50 µg/mL streptomycin, which is consistent to Champion and coworkers paper (1988). Strain B199 also grew under streptomycin presence. Hence 0.5% LiCl, and 0.5% propionic acid would be useful as selective agent for *R. albus* isolation, and nalidixic acid, would be evaluated in less concentration in strain 8.

Table 1: *R. albus* strains screening resistance to selective agents after 24 hours of incubation

Selective agent	Strains			
	R. albus 7	R. albus 8	R. albus SY3	R. albus B199
LiCl (0.5%)	+	+	+	+*
NaCl (5%)	-	-	-	-
Propionic acid (0.5%)	+	+	+	+*
Nalidixic acid (25 µg/mL)	+	-	+	+
Streptomycin (50 µg/mL)	+	-	-	+
Gentamicin (5 µg/mL)	+	-	-	-
Polymixin B (15 µg/mL)	-	-	-	-

*Growth was observed after 48 hours of incubation

Figure 9. Growth curve of *R. albus* strains incubated at 37°C (green or blue line) and at 39°C (red line). a) Strain 7, b) strain 8, c) strain SY3 and d) strain B199.



4. Discussion

Ruminococcus albus could not be obtained throughout the Hungate methodology using the RGC-modified culture medium. For instance, the culture media preparation, growth, and cryopreservation of *R. albus* strain 7 was carried out following the reconditions of the DSMZ and the protocols of the IBUN and USBA labs. Later, with the R. albus strains 7, 8, SY3 and B199 of *R. albus* following the recommendations and methodologies of The Animal Sciences Laboratory at the University of Illinois at Urbana-Champaign. In the first step of this work, a total of 161 isolations were obtained from individuals of five Colombian beers of bovines; 62, from the medium using CMC as carbon source and 99 from the medium using PMC as carbon source. The microscopic morphology of these isolation was diverse; cocci, rods, spiral shape and “S” shape were observed. 12 presumptive pure isolations which cells shape was cocci, were cryopreserved. When they were revived, just four grew again and three of these four could be identified by 16s rRNA gene Sequencing. All three strains were isolated from CMC culture medium and were identified as *Streptococcus bovis/Streptococcus equinus* instead of *Ruminococcus albus*. Hungate method undoubtedly allows isolation of cellulolytic rumen fluid bacterial species, but using CMC or PMC as the only selective agents is not specific enough to isolate a target cellulolytic specie or group of close-related cellulolytic bacterial species.

Nyonyo and coworkers (2014) evaluated the isolation of rumen cellulolytic bacteria using a new culture medium proposed by the same research group (Nyonyo *et al.,* 2013). Nyonyo research group identified by sequencing of 16s rRNA gene, isolations of rumen bacteria using Whatman N°1 filter paper (PMC), Carboximethyl-cellulose (CMC) and xylan as carbon sources, both in combinations and each one alone. They obtained a total of 129 isolations belonging to six phyla: *Firmicutes, Proteobacteria, Spirochaetes, Bacteroides, Fibrobacteres,* and *Actinobacteria*. The isolations exhibited the capability to degrade the three carbon source, two or just one (Figure 10). In a qualitative scale from 1 to 5, isolations related to genus *Ruminococcus, Fibrobacter, Pseudobutyrivibrio, Lechnospiraceae, butyrivibrio, Streptococcus, Enterococcus,* and *Prevotella* degraded filter paper in a value of 2. In a value of 3 of degradation, isolations were related to genus *Ruminococus*. According to results of this study more than the traditional three rumen fluid predominant-believed species of bacteria *Fibrobacter succinogenes, R. albus* and *R. flavefaciens*

(Russell *et al.,* 2009) were able to grow using filter paper as sole carbon source. Moreover, the genus identity could be stablished just for 19.4% of isolations. which suggest, still there is a lot of non-identified fibrolytic rumen microorganisms.

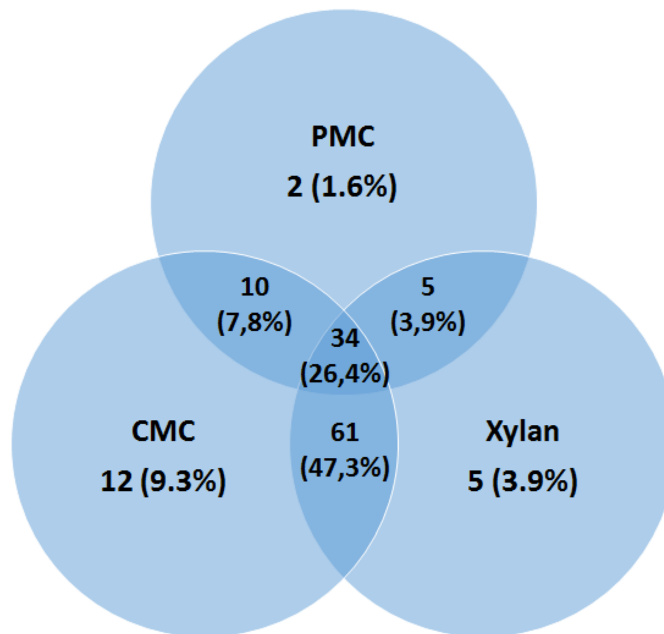


Figure 10: Diagram which represents the isolations obtained by Nyonyo and coworkers (2014) grouped by their degradation capability of PMC, CMC and xylan. Several isolations are able to degrade two or even the three carbon sources. Modified from Nyonyo and coworkers (2014).

In Nyonyo's study it also can be highlighted that not all isolations able to degrade CMC are able to degrade PMC which suggests that the use of only PMC as both carbon source and selective agent narrows the spectra of species which could be isolated and avoid the isolation of undesired species such as *Streptococcus bovis/ Streptococcus equinus*. *Streptococcus bovis* nor *S. equinus* are fibrolytic microorganisms but are amylolytic (Theodorou and France, 2005). They could grow in CMC medium because they probably have β-D-glucosidase activity (Lynd *et al.,* 2002). CMC is a cellulose soluble derivate generated by C2, C3 and C6 carboxymethyl substitutions in the glucose units (Gelman, 1982). Microorganisms that grow in CMC produce endoglucanases in absence of activity against cellulose, which means that it cannot be assumed these microorganisms have

cellulolytic activity (McDonald *et al.,* 2012). For instance, although CMC is a suitable to use both in solid and liquid culture media, is not confident for isolation of only cellulolytic bacteria. Additionally, despite in four of the five Colombian breed of cattle, the most of the bacteria were recovered from PMC actually non fibrolytic-degrading cocci could be identified in this attempt.

Another concern that may explain why *R. albus* could not be isolated would be related to its abundance in the rumen fluid. It has been estimated that this bacteria represents among 20% to 10% of the culturable isolations (Varel and Dehority, 1989), and the 2.2% of the total of the microorganisms populations (Kraus *et al.,* 1999), and it has been cultured since 1957 (Hungate, 1957). From these numbers it can be inferred that is not impossible to isolate nor cultivate *R. albus*. Therefore, failure in the attempt of isolation not only depends on the randomly matters, but also in the culture media and methodologies. The first issue to be highlighted is that before conducting the isolation assays, the growth of any *R. albus* reference strain in the RGC-based media should be evaluated. So it would be useful to know previously if *R. albus* really grows on these culture media.

Both culture media key components and procedures in *R. albus* isolation, growth, manipulation and cryopreservation in which there was inconsistencies along this work were recognized in the step of evaluation of growth of the four *R. albus* strains in the chemical-defined medium. These issues are discussed below and highlighted as recommendations.

4.1 Recommendations

- Isolation of rumen cellulolytic microorganisms by Hungate method:

McDonalds and coworkers (2012) recognize Hungate method as the best method developed for isolation and cultivation of anaerobic microorganisms which cannot be cultured in Petri plates. But they also remark researchers usually have difficulties when colonies are taken off the rolled tubes because micro-colonies adjacent to cellulolytic colonies can be also taken. In the first step of this work, in 89 of 161 colonies more than one morphology was observed under microscope. To avoid remove contamination an

assure purification in isolations, McDonalds and coworkers (2012) suggest a procedure which is analogous to Koch's postulates to confirm that isolations really are cellulolytic. In a first step, transfer the colony from the solid medium to serial dilutions solid medium containing just a sugar as carbon source, for example, cellulose. From the las dilution take just one colony and inoculate again in serial dilutions medium, and repeat this procedure one time more till colonies of the same morphology grow. Then transfer one of the colonies to a cellulose containing serial dilutions solid medium. So it is expected that colonies whit cellulolytic activity grow.

- Sampling and extraction of rumen fluid for isolation of microorganisms:

The correct sampling of rumen fluid for isolation of microorganisms is a crucial step for successful isolation. Despite in the trait of the present study isolations could be obtained, sampling of rumen fluid was not correct at all because oxygen exposure and contamination with other fluids such as saliva must be avoided as much as possible. This procedure has two key steps: the collection of rumen fluid and the isolation of rumen bacteria and other cells.

For rumen collection is desirable to use fistulated animals. First a sample of rumen is taken with the hand from the inferior part of the rumen. Filter the sample with four cheesecloth disposed over a bottle previously heated at 39 °C. Discard the solid material and continue this procedure till obtain the needed amount. Filter again the contents through a cheesecloth. For bacterial isolation, dispose the collected rumen fluid into a separatory funnel, gasify with CO2 and incubate at 39 °C for 20 minutes. Discard the inner layer of the funnel to avoid the protozoa presence and big food particles. Measure the rumen fluid final volume and dilute with McDougall's buffer in a proportion of 1:4. 1 litter of McDougall's buffer is composed by: sodium bicarbonate 9,8 g, sodium phosphate dibasic 2,44 g, potassium chloride, 0,57 g, magnesium sulfate 0,12 g and calcium chloride 0,16 (Laboratorio de Nutrición Animal, 2016).

Table 2: Components of culture media expressed in percentage used for *R. albus* grow traits

Components		Culture media		
		RGC	DSMZ 436	Chemical-defined medium
Mineral solution I	**K_2HPO_4**	0.09	0.024	0.024
Mineral solution II	**KH_2PO_4**	0.09	0.024	0.024
	$(NH_4)_2SO_4$	0.18	0.08	-
	NaCl	0.18	0.048	0.048
	$MgSO_4 *7H_2O$	0.018	0.01	0.0048
	$CaCl_2*7H_2O$	0.018	0.0064	0.0048
Volatile Fatty Acids solution	**n-valeric acid**	-	-	0.011
	isobutyric acid	0.008	0.01	0.011
	isovaleric acid	0.008	0.01	0.011
	2-methylbutyric acid	0.008	0.01	0.0094
	Acetic acid	-	-	0.137
	Propionic acid	-	-	0.06
	Butyric acid	-	-	0.0368
	PPA	-	-	0.00374
	PAA	-	-	0.0034
Haemin solution	**Haemin**	-	-	0.00005
Nitrogen source	**$(NH_4)_2SO_4$**	0.18*	0.08*	0.4
	Tryptone		0.5	-
	Yeast extract	0.5	0.2	-
Carbon sources	**Glucose**		0.3	-
	cellobiose	0.2	0.2	0.4
	Carboxymethyl-cellulose CMC)	1**	-	1**
	Pebble milled cellulose (PMC)	1**	-	-
	Microcrystalline cellulose	-	-	0.4**
	AHP-CS	-	-	0.4**
Buffer	**$NaHCO_3$**	1.2	-	0.4
	Na_2CO_3		0.4	-
Reducing agents	**$Cysteine\text{-}HCl*H_2O$**	0.2	0.05	0.1
	$Na_2S.9H_2O$	-	-	0.1

*$(NH_4)_2SO_4$ is included in mineral solution I

**These carbon source are used when fibrolytic activity want to be evaluated or fybrolytic strains want to be isolated.

- pH of the culture media should be adjusted to 6.8-7.0 under oxygen-free 100% CO_2 gas stream:

K_2HPO_4 and KH_2PO_4 salts work as a buffer in culture medium preventing from abrupt changes due to metabolic products (McDonald *et al.,* 2012). Sodium carbonate or sodium bicarbonate, in combination with CO_2 gas under anaerobiosis, maintains a stable pH of the culture medium as a second buffer (McDonald *et al.,* 2012), resembling nature's buffer carbon dioxide – carbonate and bicarbonate (Wolfe *et al.,* 2011). The presence of CO_2 in

the rumen due to fermentation and the bicarbonate and phosphate salts contained in the saliva of the animal which allows maintain the rumen pH in 6-7 values (Theodorou and France, 2005). When nitrogen or hydrogen is present in high concentration in the gas stream, the principal buffers are K_2HPO_4 and KH_2PO_4 (McDonald *et al.,* 2012).

For this reason, it is important to adjust the pH under CO_2 stream. DSMZ suggest in their cultivation of anaerobes manual (available in https://www.dsmz.de/) to maintain the same concentration of CO_2 in the streaming in all the steps of culture medium preparation. In other words, to use the same gas concentrations in all the steps of the culture media preparation and manipulation whether it would be work using both gas streaming station and anaerobic chamber or just one of both. When the CO_2 concentration is changed in any step, final pH won't be the same than it presumptively was adjusted. For this reason, *R. albus* growth would be inhibited when it was intended to revive in both traits carried on with the strain 7 provided by DSMZ.

On the other hand, in the Ntaikou and coworkers (2009a) study, when *R. albus* was cultured in continuous at acid pH values (amongst 5.9 and 6.8), the stoichiometric proportions of the products were the same than those obtained by Iannoti and coworkers (1973) in batch cultures (equation 1). They also evaluated the metabolites production at these low pH values, and at high hydrogen partial pressure finding that ethanol and acetate proportions didn't change. They concluded that this stoichiometric change also occurs when external medium turns acid as a mechanism of the cell to delay acidification because instead of 4 mole of acetate, it is going to be produced 2 mole of acetate and 2 mole of ethanol. Therefore, the pH of any medium for *R. albus*, should be adjusted and remain close to the neutral value (6.8-7.0) to get an optimal growth and ATP production at least in exponential growth phase.

- Carbon sources:

As carbon sources, the most of *R. albus* strains are able to break down cellulose (Pavlostathis *et al.,* 1988), hemicellulose and xylan (Dehority, 1973), and asimílate and metabolice glucose, cellobiose, xylose and arabinose (Ntaikou *et al.,* 2008; Thurston *et al.,* 1993; Thurston *et al.,* 1994). But it has been found that among strains metabolism capabilities differ. For example, strain 7, is able to break down very well cellulose,

hemicellulose and xylane, in overall term is a fibrolytic strain (Dassa *et al.,* 2014). Meanwhile, strain 8 just degrades hemicellulose (Moon *et al.,* 2011), strain SY3 also degrades cellulose as strain 7 (Wood *et al.,* 1982). But strain B199 is not cellulolytic strain (Thurston *et al.,* 1993), moreover phylogenetically is located in a basal position with respect to the other strains (Krause *et al.,* 1999). Carboxymethyl-cellulose (CMC), pebble milled cellulose and microcrystalline cellulose are complex carbon sources which allows growing just organisms which are able to degrade them. But it is suggested to use 0.05% cellobiose to allow bacteria have starting energy for expression and production of enzymatic degrading complexes. *Ruminococcus albus* grows better using cellobiose as carbon source more than glucose (Thurston *et al.,* 1993). Cellobiose would be added at 0.4% as in chemical-defined medium. The presence of only cellobiose at 0.4%.

- Nitrogen sources:

R. albus is able to use ammonia (Nolan and Dobos, 2005; Taya *et al.,* 1980), yeast extract, pancreatic digestion of casein and casamino acids (trypticase) (Bryant and Robinson, 1961). But it prefers consume ammonia and urea, more than aminoacids and peptides (Kim *et al.,* 2014). Ammonia is not included in the salt solution II of the chemical-defined medium (table 2), it is added independently during culture medium preparation in a concentration of 0.4%. *R. albus* is able to grow only in ammonia at 0.4%.

- Volatile fatty acids (VFAs):

R. albus uses VFAs such as isovaleric acid, isobutyric acid, and 2-methylburitic acid to synthetize amino acids (Allison and Bryant, 1963). It also has been reported the use of m-butyric acid, propionic acid and valeric acid (Nyonyo *et al.,* 2014) for rumen fluid bacterial growth. It would be expected that *R. albus* grows better in chemical-defined medium because chemical-defined medium has the presence of seven VFAs in contrast to the other two media which have only three VFAs. In composition is more closed than the others to the chemical composition found in rumen fluid.

- Vegetal phenolic compounds:

It has been reported some phenolic compounds that are present in the vegetal materials which decrease lag phase time and increase growth rate of *R. albus* using cellulose as carbon source, and other ones which perform a contrary effect. These are syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) that inhibits *S. bovis* growth in concentration above 4% (O'Donovan and Brooker, 2001), p-hydroxibenzoic acid, and 3-phenylpropionic acid. According to literature review, 3-phenylpropionic acid seems to be the best for improve cellulolytic activity of *R. albus* for its specificity for *R. albus* and *R. flavefaciens* (Borneman *et al.,* 1986), evaluated 3-pheylpropionic acid in the presence of cellobiose as sole carbon source but they didn't found any significant difference in growth, so they postulated that 3 – phenylproionic acid just facilitates *R. albus* adhesion to cellulose. In other studies of 3-phenylpropionic acid effect in cellulose presence, it has been reported that a set of factors increases or improves, such as the size of the capsule (glicocaliz), high molecular weight molecules (Stack and Cotta, 1986), the affinity constant (k_s) of *R. albus* for cellulose (Morrison *et al.,* 1990) and the hydrolysis (Hungate and Stack, 1982). Moreover, it is expected that the presence of 3-phenylpropionic acid and of 3-phenylacetic acid in the chemical defined medium would increase the cellulolytic activity, thus the growth of *R. albus* using complex carbon sources, more than in the other two media. For this reason, this component is advisable to be used in culture media for *R. albus* especially when cellulolytic or fibrolytic activity is going to be evaluated.

- Reducing agent:

Due to low redox potential present in the rumen (between -300 and -350) (Theodorou and France, 2005) it is advised to use agents which allow culture media to reach and maintain a low redox potential. Cystein – HCl is utilized to reduce the oxide-reduction potential by trapping oxygen molecules (Caldwell & Bryant, 1966).

- Rumen fluid presence:

Rumen fluid is a complex substance composed by 246 identified metabolite species and probable more uncharacterized ones (Saleem *et al.,* 2013). As a chemical defined medium

cannot supply all those components, microorganisms may grow better adding clarified rumen fluid.

- Culture medium head space

It is important to let at least 50% of headspace of the total of capacity of the recipient (tube, bottle, vial, etc.) in which culture medium is going to be prepared to avoid a fast increase in atmospheric pressure and metabolic gas products accumulation such as CO_2 and hydrogen. The reason not only remains in the security of the lab stuff and the personal, but also in the quality of the experiment and because it has been demonstrated that when hydrogen partial pressure increases, cell growth would be stopped as it was exposed before. The events related to hydrogen partial pressure in the rumen cavity are interesting. In rumen fluid it could be found syntrophic microorganisms which are removing the hydrogen produced by *R. albus* and by other hydrogen producing species continuously. For example, methanogenic and sulfate reducing ones. Therefore, it is expected in healthy ruminants, hydrogen partial pressure in the rumen remains low. When hydrogen partial pressure increases, it seems hydrogen-consumers' metabolism is stimulated while, hydrogen-producers such as *R. albus* metabolism is disfavored (Stams and Plugge, 2009). Furthermore, it has been found that when hydrogen partial pressure increases, formate is going to be accumulate into the cell because formate-dehydrogenase enzyme will be product-saturated (Ntaikou *et al.,* 2009a).

- Vitamins and supplements:

Vitamins and supplements such as hemin, menadione (K3 vitamin precursor), B complex vitamins (thiamin, riboflavin, folate and the cobalamin) stimulates *R. albus* growth (Bryant and Robinson, 1962; Scott and Dehority, 1965).

- Incubation temperature:

Some works report their experiments incubating *R. albus* at 37 °C, while others at 39 ºC. In this study it could be observed that despite growth curves show a better growth at 39 °C than at 37 °C, both temperatures are useful to carry on studies on *R. albus*. The growth curve of strain 7 at 39 °C has a slight higher slope than at 37 ºC but the maximum OD in

both curves is just the same. Strain 8 growth curves shows a similar tendency than ones belonging to strain 7. Growth curves of strain SY3 shows a different tendency than these of strains 7 and 8. Despite strain SY3 reach exponential phase faster at 37 °C than at 39 ºC, as slop as maximal OD are slightly higher than these at 37 ºC. These results were expected and are consistent with the fact that rumen temperature use to be among 38 °C and 42 °C because of fermentation and homeothermic metabolism of ruminants (Theodorou and France, 2005).

- Cryopreservation

According to results of this work, cryopreservation of *R. albus* strains would be better if it is performed in serum bottles agar slants more than in cryo-tubes. When cryo-tubes were subtracted from the -20 ºC freezer in the *R. albus* isolation trait of this work, the color of the medium was pink, which means oxygen was present. It would be the explanations for the results obtained when the revival attempt was done. Just four of the nine strains that wanted to be sequenced could grow again and the three that could be identified were *Streptococcus bovis* and *Streptococcus equinus*; both are anaerobic facultative bacteria for instance resists oxygen presence (Romero-Hernández *et al.,* 2013). For this reason, agar slants in serum bottles is a better method for cryopreservation of strict anaerobes.

- Reactivation

DSMZ in their manuals recommend for anaerobic bacteria to carry on reactivation of lyophilized microorganisms in presence of oxygen-free sterile gas streaming or under anaerobic atmosphere using an anaerobic chamber and working with syringes and needles. Oxygen exposure would not warranty a good recovering. DSMZ also affirms that frequently the freeze dried cultures of anaerobic strains takes a long lag phase till rehydrate and grow. This may explain the reason because in both trials of reviving the DSMZ *R. albus* strain 7 it took 9 days to revive.

Moreover, from the experience of this work it would be suggested that when culture which is going to be reactivated is stored in serum bottle agar slant, try to take the most of liquid

that syringe allows absorbing it from agar, it would warranty a more probable successful recovering.

- Storage of working cultures:

Additionally, it is important to remark that the fact that the 34% of the isolations in the first step of this work could not revive after store at 4 °C in the first stage of this work. Something similar occurred in the last stage when the work with the four strains was carried out. After storing at 4 °C and be reinoculated, they didn't grow again. This suggest that not always is confident to store working culture of microorganisms, it is better to maintain working cultures at 37 °C or 39 °C, preparing new inocula among every two days to once a week.

- Evaluation of inhibitory effect in *R. albus* of further selective agents.

Some of the selective agents listed in the introduction of this work could not be evaluated in the present study. For instance, in future studies, *R. albus* resistance to the following agents in the proposed concentrations according to literature review would be evaluated: Potassium tellurite in ranges of 0.5%-2.5%, phenylethyl alcohol in ranges of 0.25%-0.5%, colisitn sulfate (polymyxin E) 3.5 μg/mL -20 μg/mL, amikacin in ranges of 0.05 μg/mL – 16 μg/mL trimethoprin in ranges of 1.5 μg/mL – 20 μg/mL, D-cycloserine in ranges of 10 μg/mL – 500 μg/mL.

5. Conclusions and future perspectives

In this work it was achieved to find a culture medium composition and the procedures for preparation of it, which mimics the nutritional and the environmental conditions that favor the growth and cryopreservation of strains of *R. albus*. The contribution of this work to the science development is the transferring of knowledge that will allow to improve and extend the study of anaerobic microorganisms in Colombian institutions, specially concerning to microbiota of the rumen fluid.

Chemical-defined medium would be an ideal medium for isolation of *R. albus* from rumen by the roll-tube technique, using pebble-milled cellulose (PMC) as carbon source if good cellulolytic *R. albus* strains want to be isolated, or vegetal fiber if the interest is on *R. albus* overall strains. The four strains of *Ruminococcus albus*; 7, 8, SY3 and B199 used in the last stage of this work were able to grow in the chemical-defined medium after less than 24 hours of incubation. Cultures reached the exponential phase at 8 – 10 hours after inoculation. In contrast to the strain 7 grown in the DSMZ436 medium, which grew 9 days after resuscitation and 48 hours further reinoculations. It would be interesting to evaluate *R. albus* growth in DSMZ436 culture medium performing the preparation procedure followed for chemical defined-medium and compare the results to find out the effect of the media composition in *R. albus* growth.

In the trait for isolating *Ruminococcus albus* from rumen fluid it was identified two isolations of *Streptococcus bovis* and one of *S. equinus* for several reasons: i. *S. bovis* and *S. equinus* have a similar morphological description than *R. albus*. ii. They could be isolated from CMC media; CMC allows the growth of microorganisms which perform β-glucanase activity but that not necessarily or actually are fibrolytic. And iii. In the cryopreservation of recovered isolations, the cryo-tubes used were not impermeable to oxygen. This step undesirably selected oxygen-tolerant isolations.

According to these issues, it would be suggested that for isolation of *Ruminococcus albus*, strict anaerobic conditions shall be maintained in all the steps of the process, as in preparation of culture medium as in manipulation of grown cultures. The anaerobic chamber use, facilitate the procedures saving reagents and time. Reagents which favor their

metabolism and growth most be added to culture media, and procedures recommended in the literature must be followed with as much as fidelity as possible. They may not be modified because final results would not be different than those expected.

Concerning to selective agents, further assays should be conducted to find such one which allow growth all the known strains of *R. albus* and which would be used as selective agents.

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7. Appendices

Appendix A: Opening of Ampoules and Rehydration of Dried Cultures (anaerobic microorganisms) recommendations of DSMZ (available in https://www.dsmz.de/)

It is important to retain anoxic conditions during all steps after the opening of ampoules with freeze-dried anaerobes.

1. Remove the glass ampoule from the secondary packaging.
2. Heat the tip of the ampoule in a flame.
3. Place two or three drops of water onto the hot tip to crack the glass
4. Carefully strike off the glass tip with an appropriate tool (e.g. forceps)
5. Remove the insulation material with forceps and take out the inner vial
6. Lift the cotton plug using a forceps, remove it, keep it under sterile conditions and flame the top of the inner vial
7. After opening keep the inner vial under a flow of oxygen-free gas by inserting a gassing cannula.
8. Add approximately 0.5 ml of the recommended anoxic medium to the vial and suspend the cell pellet completely (in some cases this may take several minutes).
9. Transfer the cell suspension either by using a 1 ml syringe with hypodermic needle, which was made anoxic by flushing with oxygen-free gas.

Remarks:

- We recommend to prepare also 1:10 and 1:100 dilutions of the inoculated medium, because some ingredients of the freeze-dried pellet may inhibit growth in the first tube. Inoculation of only one tube may prevent successful resuscitation of certain lyophilized strains (e.g., *Geobacter spp.).*
- In most cases freeze dried cultures of anaerobic strains exhibit a prolonged lag period upon rehydration and should be given at least twice the normal incubation time before regarding them as non-viable.

- An anaerobic gas chamber should be available.

It is recommended to score the ampoule with a sharp file at the middle of its shoulder about one cm from the tip.

1. Transfer the ampoule with the file mark in the anaerobic chamber and strike the ampoule with a file or large forceps to remove the tip.
2. If necessary, wrap the ampoule in tissue paper and enlarge the open end by striking with a file or pencil, then remove the glass wool insulation and the inner vial.
3. Gently raise the cotton plug and sterilize the upper part of the inner vial using an incandescent flaming device
4. (Alternatively wipe the upper part of the inner vial with tissue paper soaked in 70% ethanol).
5. Add approx. 0.5 ml of anoxic medium to resuspend the cell pellet and transfer the suspension to a vial with the recommended cultivation medium (5 to 10 ml).

Remarks:

- If possible the last few drops of the suspended cell pellet should be transferred to an agar plate or slant of the recommended medium to obtain single colonies in order to check the purity of the strain. Anaerobic incubation conditions for agar plates can be achieved by placing plates in an anaerobic chamber or an activated anaerobic Gas Pak jar or similar system.

Appendix B: protocol for genomic DNA extraction of rumen fluid samples and *R. albus* cells

A modified protocol of the PowerSoil® DNA isolation kit was used as in de following description:

1. Transfer 200 µL of the Power Beads tube liquid phase to the falcon tube with the pellet of bacterial cells and resuspend it.
2. Transfer de suspended cells to the Power Beads tube and gently vortex to mix.
3. Add 60 µL of the solution C1 and mix by inversion or vortex briefly.
4. Secure Power Bead tube to a vortex adapter and vortex at the maximal speed for 10 minutes.
5. Centrifuge tubes for 30 seconds at room temperature.
6. Transfer the supernatant to a clean 2 mL collection tube. Expect the supernatant between 400 to 500 µL.
7. Add 250 µL of solution C2 and vortex for 5 seconds.
8. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes for 1 minute at room temperature.
10. Avoiding the pellet, transfer up to but no more than 600 µL of supernatant to a clean 2 mL collection tube.
11. Add 200 µL of solution C3 and vortex briefly.
12. Incubate at 4 °C for 5 minutes.
13. Centrifuge the tubes for 1 minute at room temperature.
14. Avoiding the pellet, transfer up to but no more than 750 µL of supernatant to a clean 2 mL collection tube.
15. Add 1200 µL of solution C4 to the supernatant and vortex for 5 minutes.
16. Load approximately 675 µL onto a spin filter and centrifuge for 1 minute at room temperature. Discard the flow throughout and add 675 µL of

supernatant to the spin filter and centrifuge for 1 minute at room temperature. Load the remaining supernatant onto the spin filter and centrifuge for 1 mi8nute at room temperature. A total of three loads for each sample processed are required.

17. Add 500 µL of solution C5 and centrifuge at room temperature for 30 seconds. Discard the flow throughout.
18. Centrifuge again at room temperature for 1 minute at 10000g.
19. Carefully place the spin filter in a clean 2 mL collection tube. Avoid any solution C5 into the spin filter.
20. Add 25 µL of the solution C6 to the center of the white filter membrane.
21. Centrifuge at room temperature for 1 minute at 10000g.
22. Discard spin filter. DNA in the tube is now ready to be used for any downstream application. No further steps are required.

Appendix C: protocol for PCR products DNA purification

To purify PCR products, QIAquick® PCR purification kit of Sample and Assay Technologies was used as follows:

1. Add 5 volumes of buffer PB to 1 volume of the PCR reaction and mix.
2. Place a QIAquick column in a provided 2 mL collection tube
3. To bind the DNA, apply the sample to the QIAquick column and centrifuge for 1 minute. Discard flow through and place QIAquick column back in the same tube.
4. To wash add 0.75 mL buffer PE to the column and centrifuge for 1 minute. Discard flow throughout and place QIAquick column back in the same tube.
5. Centrifuge column once more in the provided collection tube for 1 minute to removal of residual wash hopper.
6. Place columns in a clean 1.5 centrifuge tube.
7. To elute DNA add 25 µL of molecular-grade water to the center of the column (instead of 50 µL of buffer EB), and centrifuge the column for 1 minute.