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From mouth to mammary gland: a nanotechnology application in dairy cow nutrition for targeted lysine delivery

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FROM MOUTH TO MAMMARY GLAND: A NANOTECHNOLOGY APPLICATION IN DAIRY COW NUTRITION FOR TARGETED LYSINE DELIVERY

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"The Road goes ever on and on,
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say…"

J. R. R. Tolkien, in *The Lord of the Rings: The Fellowship of the Ring*

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Dissemination

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Abstract

Lysine (Lys) is one of the most limiting amino acids (AA) in high production dairy cows fed corn-based diets. For this reason, supplementing this specific AA to the animal could improve milk production while simultaneously reducing overall feed costs. Technologies and products that focus on protecting Lys in the rumen already exist, but their efficiency in this task is not enough to adequately supply Lys to fit high production needs, mainly due to poor ruminal resistance.

In this work, a novel rumen protection technology is proposed, relying in nanotechnology to develop lipid-based delivery systems for Lys. Saturated fatty acids, particularly stearic and arachidic acid, were selected for this approach due to their natural resistance to ruminal digestion and also for their high melting points. To develop the optimal nanoparticle (NP), three different NP structures were considered in this work: solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and multiple lipid nanoparticles (MLN). A Lys quantification method, based on high-performance liquid chromatography with fluorescent detection, was also developed and validated in this project. All these NP were produced using an organic solvent-free method based on sonication and loaded with Lys, reaching Lys encapsulations of 40%, 65% and 90%, for SLN, NLC and MLN, respectively.

All the aforementioned NP were assessed in terms of ruminal digestion resistance, with SLN being found to both resist the ruminal conditions and protect their Lys content, being therefore selected for further studies. The SLN, composed of stearic or arachidic using Tween® 60 as surfactant, were optimized using a Box-Behnken design to maximize Lys content, achieving encapsulation values of 53% and 61%, respectively. Both these SLN were also able to resist the high temperatures achieved during industrial feed processing, up to 60 ˚C. Furthermore, SLN composed of stearic acid were produced using cheaper components and with an easily scalable method, based on high-pressure homogenization, without compromising their physical

properties or their ability to encapsulate Lys. These properties were also maintained over at least one month of storage.

The optimized SLN, composed of stearic or arachidic acid using Tween® 60 as surfactant, were able to resist digestion in both abomasum and small intestine mimicking media without compromising their integrity. These SLN were found not to be inherently toxic *in vitro* as well, enabling them to be considered for *in vivo* applications. Furthermore, both SLN were degraded over a period of 24 h in the blood, particularly stearic acid SLN that were completely destroyed after this period, indicating that they could release their Lys cargo once in the bloodstream to be used by the animal.

Further studies should be performed to validate *in vivo* the efficacy of the proposed SLN in delivering Lys to dairy cows, but the results present in this work indicate their potential as an alternative AA delivery system in ruminants and highlight the benefits that nanotechnology-based approaches could bring to animal nutrition and feeding.

Resumo

A lisina (Lys) é geralmente um dos aminoácidos (AA) limitantes em vacas leiteiras de alta produção alimentadas com dietas à base de milho. Por esse motivo, a suplementação deste AA específico ao animal pode melhorar a produção de leite e, ao mesmo tempo, reduzir os custos com a alimentação. Apesar de existirem formulações comerciais de lisina protegida, a sua eficiência não é suficiente para atender às necessidades de animais de elevada produção, principalmente devido uma baixa resistência à digestão ruminal.

Neste trabalho, é proposta uma nova tecnologia de proteção ruminal, recorrendo à nanotecnologia para desenvolver sistemas de transporte de Lys, baseados em lípidos. Neste sentido, foram selecionados ácidos gordos saturados, particularmente o ácido esteárico e araquídico, devido à sua resistência natural no rúmen e também devido aos seus altos pontos de fusão. Foram consideradas três estruturas de NP: nanopartículas lipídicas sólidas (SLN), transportadores lipídicos nanoestruturados (NLC) e nanopartículas lipídicas múltiplas (MLN). Foi também desenvolvido e validado um método de quantificação de Lys, baseado em cromatografia líquida de alta performance com deteção de fluorescência. Todas as NP foram produzidas usando um método sem recurso a solventes orgânicos, baseado em sonicação, e carregadas com Lys, atingindo taxas de encapsulação de 90%, 65% e 40%, para as MLN, NLC e SLN, respetivamente.

Foi avaliada a resistência à digestão ruminal das NP, tendo sido observado que as SLN eram simultaneamente capazes de resistir a esta digestão e de proteger o seu conteúdo de Lys, tendo sido, por isso, selecionadas para os estudos posteriores. As SLN, compostas por ácido esteárico ou ácido araquídico usando Tween® 60 como surfactante, foram otimizadas usando o método de Box-Behnken para maximizar o seu conteúdo em Lys, tendo sido atingidos os valores de taxas de encapsulação de 53% e 61%, respetivamente. Ambas as SLN foram capazes de resistir às altas temperaturas, até 60 ˚C, vulgarmente utilizadas durante a tecnologia de fabrico de alimentos compostos. Adicionalmente, foi possível produzir SLN compostas de ácido esteárico

usando componentes mais baratos e com um método facilmente escalável, baseado na homogeneização a alta pressão, sem comprometer as suas propriedades físicas ou a sua capacidade de encapsulação de Lys, mantendo essas propriedades por pelo menos um mês de armazenamento.

As SLN otimizadas, compostas de ácido esteárico ou araquídico usando Tween® 60 como surfactante, foram capazes de resistir à digestão em meios miméticos do abomaso e do intestino delgado, sem comprometer a sua integridade. Constatou-se ainda que estas SLN não são inerentemente tóxicas *in vitro*, permitindo que sejam consideradas para aplicações *in vivo*. Ao fim de 24 horas de incubação em sangue bovino, ambas as SLN foram degradadas, indicando a possibilidade de libertarem a sua carga (Lys) na corrente sanguínea para uso pelo animal.

Devem ser realizados estudos adicionais para validar a eficácia *in vivo* das SLN propostas relativamente à entrega de Lys em vacas leiteiras, mas os resultados apresentados neste trabalho indicam o potencial das SLN desenvolvidas como um sistema alternativo de entrega de AA em ruminantes, destacando os benefícios que abordagens baseadas em nanotecnologia podem trazer para alimentação e nutrição animal.

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List of abbreviations

%LC	Lysine loading capacity
%Lys	Lysine encapsulation efficiency
3D	Three-dimensional
AA	Amino acid
ANOVA	Analysis of variance
AVD	Arteriovenous difference
BBD	Box-Behnken designs
BHB	Beta-hydroxybutyrate
CC	Chromatographic control
CP	Crude protein
CS	Corn silage
DDGS	Dried distillers grans with solubles
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle medium
DMI	Dry matter intake
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
ELS	Electrophoretic light scattering
ES	Extruded soybean

FaSSGF	Fasted state simulated gastric fluid
FBS	Fetal bovine serum
FeSSIF	Fed state simulated intestinal fluid
GRAS	Generally regarded as safe
GS	Grass silage
HPH	High-pressure homogenization
HPLC	High-performance liquid chromatography
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantification
LV	Latent variables
Lys	Lysine
Met	Methionine
MLN	Multiple lipid nanoparticles
MP	Metabolizable protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIR	Near-infrared
NLC	Nanostructured lipid carriers
NP	Nanoparticles
NRC	National research council
PBS	Phosphate buffered saline

PdI	Polydispersity Index
PLS	Partial least squares
R^2_{CV}	Cross-validation coefficient
RI	Recrystallization index
RMSECV	Root mean square error of cross-validation
RP	Rumen-protection
RP-AA	Rumen-protected amino acid
RP-Lys	Rumen-protected lysine
RP-Met	Rumen-protected methionine
RS	Raw soybean
RSD	Relative standard deviation
RT	Room temperature
SEM	Standard error of the mean
SLN	Solid lipid nanoparticles
SNV	Standard normal variate
T0	Timepoint equivalent to contact with the media but no incubation was allowed to occur
TEM	Transmission electron microscopy
Tf	Timepoint equivalent to the end of incubation in the media
Ti	Timepoint equivalent after nanoparticle production
VFA	Volatile fatty acids
W/O/W	Water-in-oil-in-water

ΔH	Enthalpy

Thesis Structure

This PhD thesis is organized in 8 sections. The organization and a brief description of what is discussed in each of these sections is detailed here to guide the reader.

Introduction

This section contextualizes the developed work and provides a state of the art review of the currently available rumen protection technologies. Nanotechnology concepts and applications and how they can improve the existing methods are also described in this section.

Rationale of the Thesis

The motivation that drove the development of this work is explained in this section. The objectives that derived from this motivation and that guided this study are also presented.

Chapter 1. Lysine quantification method

This section focuses on the development, optimization and validation of the quantification method used throughout this work to quantify lysine.

Chapter 2. Nanoparticle production

The main aspects of nanoparticle production and physical characterization are presented in this section. Besides the characterization of physical properties, the nanoparticles ability to resist ruminal digestion and industrial processing conditions are also assessed. After nanoparticle structure and composition selection, the production was optimized to achieve the ideal formulation. An evaluation of the feasibility of scaling production of these nanoparticles was also performed in this chapter.

Chapter 3. Nanoparticles biostability throughout the digestive tract and beyond: *in vitro* and *ex vivo* studies

This section focuses on the study of nanoparticle integrity after bypassing the rumen, both in the abomasum and small intestine and in the bloodstream. An assessment of the protection of lysine conferred by the nanoparticles in the rumen is also performed. An *in vitro* study was also performed to assess the cellular toxicity of the proposed nanoparticles.

Concluding remarks

The main conclusions and findings of this work are presented in this section, highlighting the proposed nanoparticles capabilities, advantages and limitations.

Future work and perspectives

This chapter focuses on the studies that are still required to fully validate the proposed nanoparticles and suggests how the same can be performed.

Appendix

A brief explanation of the basic principles of the techniques and methodologies used throughout this work is present in the appendix section.

Introduction

When considering lactating dairy cows, the conversion of dietary nitrogen into milk protein has a low efficiency, usually ranging between 20 and 35% [1]. This low efficiency causes a high amount of the ingested nitrogen to be excreted in feces and urine, which usually brings negative impacts to environment [2-4].

Studies have found that reducing the dietary protein content can improve the conversion efficiency. However, this can compromise milk production and milk protein yield [5-8] as the amino acid (AA) profile that is absorbed in the small intestine is also a determinant factor of milk protein synthesis and nitrogen efficiency [4, 9]. Therefore, dietary supplementation with individual AA constitutes a strategy to balance the AA profile of the metabolizable protein (MP), which enables to decrease protein intake without compromising milk output [8, 10, 11].

Lysine (Lys) and methionine (Met) are often found to be the most limiting AA for milk protein synthesis in cows fed corn based-diets [12]. Several studies have been performed to determine the most adequate amount of these two AA for milk production but also to improve their supply [1, 2, 4, 10, 13-15]. According to the National Research Council [12], for an optimal MP use, the percentages of Lys and Met in the MP should be 7.2 and 2.4%, respectively. It has also been found that more important than the individual values of these AA is their ratio of 3:1 (Lys:Met), crucial to improve milk production efficiency [2, 9, 12, 15]. An efficient strategy to achieve the recommended amounts of these AA, without greatly increasing protein intake, could derive from using rumen-protected (RP) AA [16-19]. Such approaches could enable an increase of specific AA available for intestinal absorption but remove the need to considerably alter the crude protein (CP) of the diets [20-22]. Several RP-Lys and Met are already commercially available, but their effects on milk yield and composition have been variable and inconsistent, particularly when considering RP-Lys products [2, 23-26].

The following sections present the technologies behind the currently available commercial RP-Lys products and the effects observed on milk production and composition through dietary supplementation with these products. Nanotechnology will be then presented as an alternative and innovative methodology for the design of RP-AA delivery systems.

Existing rumen-protection technologies

The protection of AA from digestion in the rumen has been sought mainly through entrapping them inside rumen-resistant shells that are inert in the rumen, being destroyed in the posterior digestive tract, releasing their content to be absorbed in the small intestine.

Some of the first strategies to attempt ruminal protection relied on coating an AA core with pH-sensitive polymers. These shells were designed to protect their cargo in near neutral pH of the rumen but dissolve in the acidic pH of the abomasum, releasing their content [27, 28]. They function by taking advantage of structural changes created by different pH values in polymeric matrices. They create a stable and continuous film around its content at near neutral pH, but destabilize at low pH values, thus exposing their AA core and enabling their release for intestinal uptake [27, 28]. *In vitro* studies performed with these RP-AA indicate that the conferred protection was very high (up to 95% of the AA resisted ruminal digestion) and that the AA release in the abomasum was also high (with up to 90% of the AA content becoming available) [17, 26, 29-31]. However, both the polymers, such as styrene/vinylpyridine copolymers or cellulose propionate 3-morpholinobutyrates, and the coating method represent a considerable price. Additionally, the latter requires organic solvents [27, 28], which are in theory completely removed by evaporation, but increase not only the cost but also production time of these products.

More recent approaches rely on lipids, such as saturated fatty acids, that are naturally resistant to the microbial digestion that occurs in the rumen to form shells around a chosen AA core [22, 32-37]. These lipids coat the AA and create

a physical barrier around the latter, which protects its content by preventing access by ruminal microbiota [38]. These lipid coating-based RP products are usually cheaper than the polymeric counterparts, due to the availability of low-cost feed-grade lipids, but suffer from reduced payloads and limited post-ruminal release [39]. The latter is due to the fact that these lipids suffer very little digestion after clearing the rumen, thus maintaining always some of the coating intact, preventing the release of their contents [40-42]. Studies with these lipid-based coating have also indicated that conferred protection varies with the AA that is being protected, revealing inferior efficiency for Lys when compared with Met [43].

An *in vitro* study that evaluated a combination of pH-sensitive and lipid-based matrices [44] showed that this combination presented a high digestion resistance and improved the post-ruminal release profile, due to the presence of pH-sensitive polymer in the matrix [44].

Encapsulating AA within a rumen-resistant shell, be it a pH sensitive co-polymer or a lipid matrix, is the most commonly used method for ruminal-bypass. It is not, however, the only proposed method to achieve ruminal protection.

The pelletizing of premix concentrates supplemented with AA has also been considered and appeared to be beneficial towards milk production [45]. In this approach, a small amount of concentrate was supplemented with both Lys and Met and afterwards pelletized. The basis for this strategy was the fact that pelletization of substances increases their rate of passage in the rumen [46] , decreasing, in theory, the degree of digestion which they suffer. This method does not require any kind of coating, thus reducing the cost when compared with those alternatives and also enabling a more efficient storage and distribution [45]. However, since this approach relies on increasing the rate of passage of the AA instead of protecting it, its efficacy should be lower than other RP technologies and will always be subjected to any variations in ruminal clearance.

Instead of protecting the AA across the rumen, the use of AA-derivative compounds has also been considered. The calcium salt of hydroxymethyl Lys is a derivative of Lys produced by reacting Lys-HCl with formaldehyde in an aqueous solution of calcium hydroxide [47]. This salt has been suggested as an alternative supply of RP-Lys as it is naturally resistant to microbial digestion and it is capable of releasing free Lys in acidic media, such as that found in the abomasum [47]. Studies performed in dairy cows were inconclusive [48], due to the use of acidic silage in the diet that may have caused an early release of Lys once mixed, but found it to be effective in increasing Lys in the plasma of sheep [47]. Nevertheless, the process of producing this alternative Lys form is considerably expensive and its use should be limited to nonacidic diets.

The latter two approaches (pelletizing AA-enriched concentrate and using AA-derivatives) show potential in ruminal protection applications but they were evaluated in few studies, whereas the encapsulation of AA, be it in pH-sensitive polymers or lipid matrices, has already rendered several products that are currently available on the market. Table 1 presents several studies that have been performed to evaluate the effects of these products, particularly of RP-Lys, on feed intake, milk production and composition.

Table 1 – Studies performed to evaluate the effects of feeding commercial rumen protected lysine (Lys) products (RP) on feed intake and milk production and composition of dairy cows.

RP technology	Diet	Supplemented*		DMI[a] (Kg/d)	CP[b] (%)	Milk			Reference
		Lys (g/d)	Met (g/d)			Yield (Kg/d)	Protein (%)	Fat (%)	
pH-sensitive polymer coating	Control			19.5	14.6	26.5	3.08[a]	3.74	[26]
	RP		9	18.6	14.6	26.0	3.03[b]	3.72	
pH-sensitive polymer coating	Control			21.5	14.5	30.3	3.03	3.46	[29][#]
	RP	7.8	4.5	21.8	14.5	29.9	3.07	3.54	
	RP	18.0	10.4	21.7	14.5	29.9	3.13	3.46	
	RP	28.2	16.3	22.3	14.5	30.3	3.15	3.62	
	RP	7.8	16.3	21.1	14.5	28.3	3.07	3.53	
	RP	28.2	4.5	22.2	14.5	30.3	3.09	3.57	

pH-sensitive polymer coating	Control			23.5	16.0	42.9[a]	2.90[a]	3.69	[49]
	RP		10.5	23.1	16.0	42.7[a]	2.91[a]	3.63	
	RP	16	10.2	24.3	16.0	44.0[a,b]	2.93[a,b]	3.74	
	Control			23.9	18.5	43.4[c]	2.80[a]	3.62	
	RP		10.5	22.6	18.5	40.9[b]	3.01[b]	3.88	
	RP	16	10.2	24.2	18.5	45.8[a,b]	2.94[a]	3.65	
pH-sensitive polymer coating	Control			22.2	16.4	25.3	3.15[a]	3.65	[50]
	RP	40	15	22.2	16.4	26.3	3.25[b]	3.50	
pH-sensitive polymer coating	RS[c]			18.8	16.6	28.8	2.74	3.14[a]	[51]
	RS – RP	4.00	1.51	18.9	16.5	29.1	2.73	3.01	
	ES[c]			18.8	16.5	29.7	2.73	2.95[b]	

	ES – RP	4.00	1.51	18.8	16.4	29.4	2.74	3.08	
pH-sensitive polymer coating	Control			23.7[a]	18.3	34.2[a]			[52]
	RP	6.0	10.9	22.3[b]	16.7	32.8[b]			
	RP	7.5	7.15	22,1[b]	15.3	32.8[b]			
pH-sensitive polymer coating	GS[e]		9.1	18.7[a]	21.5	32.8	3.10	3.42	[53]
	GS – RP	31	18.4	17.8[a]	22.4	33.7	3.15	3.06	
	CS[f]		9.1	16.6[b]	26.1	31.4	3.10	2.89	
	CS – RP	31	18.4	16.3[b]	27.3	32.4	3.05	2.70	
Lipid coating	Control			20.0	15.0	32.3	3.29	3.31	[20]
	RP	4.20		20.0	15.2	33.2	3.25	2.95	
	RP	4.12	1.37	19.7	15.0	32.5	3.24	3.18	
	RP[1]	4.10	1.37	19.5	15.4	31.2	3.28	3.41	

Lipid coating	Control		16	25.4	16.6	27.1	3.41	3.70	[35]
	RP	42	16	25.1	16.7	26.6	3.48	3.73	
Lipid coating	Control			27.7	14.5	38.2	3.00[a]	3.72	[54]
	RP	27.6		28.1	14.5	37.9	3.13[b]	3.84	
	RP[2]	27.6	17.8	28.5	14.5	39.6	3.14[b]	4.01	
Lipid coating	Control			26.4	16.0	46.4	2.92[a]	3.59[a]	[55]
	RP	13.2		25.7	16.1	45.8	2.94[b]	3.61[b]	
	RP[3]	10.6		26.6	16.4	46.6	2.93[b]	3.61[b]	
Lipid coating	Control			25.9	13.7	40.3	2.84	3.47	[21]
	RP	24		26.5	13.7	41.5	2.86	3.30	
	RP	24	15	25.9	13.7	40.2	2.87	3.41	
	RP	12	9	18.9	14.6	27.9	3.11	3.63	
	RP	24	9	19.2	14.6	26.7	3.19	3.58	

Lipid coating	Control			21.0	16.3	20.7	3.74	4.18	[56]
	RP	2.5	1.0	20.4	13.7	20.5	3.71	4.25	
Lipid coating	DDG10[g]		7.4	25.9[a]	16.8	30.2	3.23[a]	3.65	[57]
	DDGS10 - RP	14.6	6.9	24.3[a]	16.8	30.3	3.23[a,b]	3.72	
	DDGS20[h]		7.4	25.7[b]	16.7	31.8	3.21[a]	3.73	
	DDGS10 - RP	14.6	7.4	25.8[a]	16.7	31.1	3.17[b]	3.78	
Lipid coating	Control			21.7	3	37.0	3.09[a]	3.26	[58]
	RP	27	8	22.8	3	41.1	2.98[b]	3.26	
	RP	40	13	22.0	3	40.7	3.12[a]	3.32	
Lipid coating	Control			21.1	17.2	38.0	3.17[a]	3.54	[59]
	RP	16	6.5	20.6	16.4	38.3	3.23[b]	3.65	
Lipid coating	Control			19.9	16.7	40.5	2.75	4.20	[60]

	RP	10	5	21.7	16.7	42.4	2.62	3.95	
Lipid coating	Control			23.3	14.0	33.9	3.21	3.79	[36]
	RP	21.1		23.0	14.0	33.5	3.21	3.80	
	RP	22.1	5.9	23.3	13.9	33.9	3.26	3.85	
Lipid coating	Control			26.2	16.8	38.3	3.16	3.61[a]	[61]
	RP	41		26.1	17.0	39.1	3.14	3.82[b]	
Lipid coating	Control			22.9	13.5	35.2[a]	2.94	3.51	[34]
	RP	24.3	18.4	23.6	13.7	36.9[a,b]	2.99	3.32	
	RP[4]	24.3	18.4	24.2	13.9	38.5[b]	3.03	3.30	
Lipid coating	Control			13.0[a]	16.2	15.89[a]	3.05	2.40	[62]
	RP	4.42	1.98	13.4[b]	16.2	17.69[b]	3.04	2.36	
Not stated	Control				16.2	28.0	3.09[a]	3.38	[63]
	RP		7.65		16.2	27.8	3.09[a]	3.61	

	RP	5.13	7.65	16.2	29.7	3.14[b]	3.50

* Supplemented values refer to amount of AA that was estimated to be supplied at intestinal level as metabolizable AA.

Different superscript letters indicate significant differences within each study, according to the authors ($P < 0.05$)

[1] RP-Leucine was also added to supply 15.21 g/d in the intestine.

[2] RP-Histidine was also added to supply 9.3 g/d in the intestine.

[3] RP-Leucine, RP-Histidine and RP-Valine were also added to supply 5.4, 2.2 and 1.6 g/d in the intestine, respectively.

[4] RP-Histidine was also added to supply 12.4 g/d in the intestine.

[a] Dry matter intake (DMI)

[b] Crude protein (CP)

[c] Raw soybean (RS)

[d] Extruded soybean (ES)

[e] Grass silage (GS)

[f] Corn silage (CS)

[g] with 10% of dried distillers grains with solubles (DDGS)

[h] with 20% of dried distillers grains with solubles (DDGS)

[#] Lysine and lysine/methionine interaction significantly increased milk yield and the use of both RP products, together or in separate, significantly increase milk protein percentage.

The effects of dietary supplementation with encapsulation-based RP-AA on milk yields are inconsistent, with positive [29, 62], negative [52] or no effects [20, 21, 26, 34-36, 49, 50, 54-61, 63]. This inconsistency in results also occurs when considering milk protein percentage, with positive [29, 34, 49, 50, 54, 55, 59, 63], negative [26, 57, 58] or no effects [20, 21, 35, 36, 51, 56, 57, 60-62] being reported.

Furthermore, some of these studies assessed the effects of feeding RP-Lys on blood Lys levels and the results were also inconclusive, with both positive [21, 34] and no effects [20, 57] being reported.

The conflicting results can be largely explained by the different conditions in which the studies were performed, namely milk production level, stage of lactation, diet composition, inclusion level of the product or even handling and manipulation of the RP products prior to animal consumption (which could decrease the efficacy of the product) [2, 23-25]. Some studies also report the low AA protection conferred by these RP products [55, 64] with degradation of more than 50% of the Lys content being registered [61]. Besides the difficulty in comparing results due the different conditions of the studies, the RP-Lys products themselves could also suffer from limitations resulting from reduced ruminal protection [18, 22, 24, 64]. The use of different tests to evaluate the Lys bioavailability of these products further increases their inconsistencies [65, 66].

The existing RP products focus solely in increasing the amount of Lys that resist ruminal digestion and not on delivering it to the bloodstream. Once bypassing the rumen, the product must be degraded or release its Lys content so it can be absorbed in the small intestine and be used by the animal. The latter is not always guaranteed and is an important concern when considering the lipid-based encapsulation [39].

It can be observed (Table 1) that, despite the results not always being reproduceable, there is a tendency towards improvement when using RP-AA, indicating that the need for newer and better RP products exists and that they can improve dairy cow farming.

Nanotechnology

Other RP possibilities could arise from fields such as nanotechnology, which has already been applied in other areas regarding transport and delivery of specific molecules. This approach, often referred to as nanodelivery, is very common in nanomedicine and numerous applications already exist [67-76]. However, the applications of nanotechnology in animal science are still very scarce [77, 78] but the existing work in other fields has proven that it can bring great benefits to animal science, particularly in rumen-bypass.

Nanotechnology is the application and manipulation of materials in the nanoscale. These materials exhibit different properties from their bulk counterparts mainly due to their very high surface-area-to-volume ratio [69, 74, 76, 79]. Nanomaterials exist in all shapes and sizes, including nanoparticles (NP), nanosheets, nanorods, nanotubes, quantum dots, nanowires and many others.

In this work, NP will be the chosen nanostructure and, regarding biological applications, they usually have two main objectives: 1) increase the bioavailability of drugs or molecules and/or 2) reduce the side effects that a substance might have in organs other than the intended target [69, 71, 75, 79-82]. To achieve this, NP rely on their small size to increase absorption or remain in circulation for longer periods of time, avoiding their payload's metabolization. Moreover, NP can also be surface-functionalized with a wide variety of agents to enhance their properties depending on their application, from concealing the NP from immune system cells to delivering them to specific tissues or cells [83-87].

These approaches can be easily applied to other areas, relying on the same mechanics and principles, to deliver specific molecules across several different membranes and/or resisting adverse conditions, rendering a countless number of diverse applications.

Lipid NP

Saturated fatty acids are able to traverse the rumen virtually unscathed and, for this reason, lipid-based nanotechnology becomes a very interesting and promising field for rumen-protection approaches.

Fat nanoemulsions were firstly developed in the 1950's as parenteral drug delivery systems. However, the properties of these emulsions were found to often lead to rapid drug releases and favored drug oxidation. Nonetheless, the use of lipids for drug delivery applications was considered very promising mainly due to their biosafety properties. This potential generated studies and works, which eventually lead to the development of several lipid-based NP with different composition and structures (Figure 1) [88, 89].

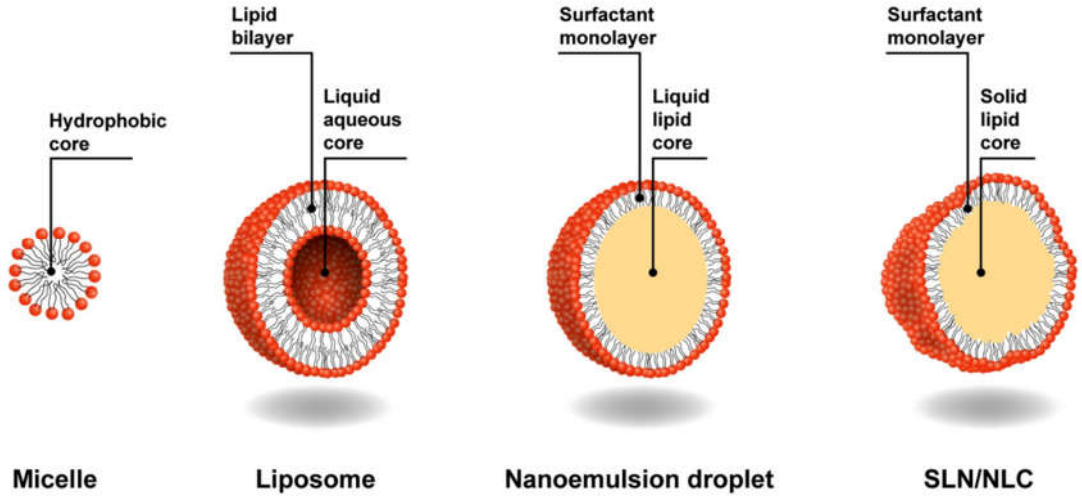

Figure 1 – Graphical representation of different lipid nanoparticles (NP): micelles, liposomes, nanoemulsion droplets and solid lipid nanoparticles (SLN)/ nanostructured lipid carriers (NLC). Taken from [89].

Micelles and liposomes are usually composed of phospholipids or other amphiphilic lipids and produced by self-assembly [90]. In micelles, the hydrophobic portion of the lipids form a core with the hydrophilic portion facing the water and stabilizing the whole structure (Figure 1). In the case of liposomes, the lipids form a phospholipid bilayer that encircles an aqueous core (Figure 1). These structures can be of numerous sizes and sometimes can

be composed of several bilayers inside each other. Considering this structure, liposomes are capable of transporting both hydrophobic molecules (within the lipid bilayer) and hydrophilic molecules (in the aqueous core) [72, 91]. However, they are costly to produce and are not very stable [92].

Solid lipid NP (SLN) and nanostructured lipid carriers (NLC) are another kind of lipid NP that possess a solid matrix coated with a surfactant agent to stabilize and maintain the lipid core. The main difference between these two NP is that SLN are composed only of lipids that are solid at body temperature whereas NLC are composed of a mixture of lipids that are solid at body temperature and lipids that are liquid at body temperature [93]. This difference causes the matrix of SLN to be highly crystalline while the matrix of NLC is more amorphous and imperfect. A more crystalline matrix translates to more stable particles, but might also facilitate molecule expulsion due to shifts in the matrix, whereas a more amorphous matrix enables higher entrapment as the imperfections allow for higher molecule loading [94-96]. These types of particles are more suitable for hydrophobic molecules loading due to their lipid matrix, but can also be used with hydrophilic molecules [97], and are known to be much more stable than liposomes. Another advantage of these NP is that they present a simpler and more easily scalable production method [98, 99] than other lipid NP and non-lipid-based NP.

Multiple lipid NP (MLN) and SLN or NLC produced by double emulsion are very similar to the previously described NP, but possess water vacuoles inside the lipid matrix [100, 101]. These vacuoles considerably increase the amount of hydrophilic molecules that can be loaded, but usually also have a more complicated production method, which sometimes resorts to organic solvents.

All the previously described NP have their advantages and disadvantages and there is no such thing as the perfect particle, but with proper understanding of the obstacles, a specific NP can be selected and tailored to better suit each objective, from different administration routes, such as oral [102], nasal [103], topic [104], intravenous [105] and vaginal [106], to distinct target organs, like the brain [107], the lungs [108], the liver [109] and the heart [110], as well as

different diseases, among which rheumatoid arthritis [111], bacterial infections [110], cancer [112] and diabetes [113]. Applying nanotechnology allows to overcome many barriers and to reduce side effects of many therapeutic agents, surpassing other approaches in each specific case.

Nanotechnology in animal science

Nanotechnology-based approaches in animal nutrition are not very numerous, but some studies already exist, indicating the relevance of this field and the benefits that can be obtained from multidisciplinary works in animal science. Currently studies that attempted to apply nanotechnology to animal science encompass immune system modulation [78, 114-119], clinical applications [120, 121] and even in animal reproduction [122]. These studies, although not numerous and more focused on metallic NP, pave the way for future applications of nanotechnology in the field of animal science, highlighting their potentiality.

Nanotechnology-based approaches, initially designed to address issues of other fields, can be adapted and applied towards improving the efficient feeding of ruminant animals. Several studies have attempted to produce digestion-resistant capsules to protect therapeutic agents across the human digestive tract, thus enabling for agents that are traditionally used intravenously to be administered orally [102, 123, 124]. The conditions and obstacles found in the human and the ruminant digestive tracts are very different, but a similar approach could be used to protect nutrients or bioactive molecules in the rumen, by tailoring the capsules to avoid or resist microbial digestion. This could be achieved by using substances that are known to resist ruminal digestion as the building blocks for the NP. Furthermore, since smaller particles are faster to exit the rumen, the use of these delivery systems could further improve their ruminal protection.

The improvement of intestinal absorption of some biomolecules has also been the interest of study of many works, which have proven that NP were capable of greatly increasing the bioavailability of these bioactive agents [125-127]. An analogous tactic could be applied in bovine nutrition, not only to increase the

absorption of biomolecules that are poorly absorbed by these animals but also in improving the bioavailability of ruminal-protection products. If the rumen-protected capsules are themselves absorbed in the intestine it would be ensured that all protected content that escapes microbial digestion reaches the blood and is not lost due to release issues. Reaching the bloodstream does not, however, guarantee an increase in bioavailability. Nanotechnology has also been applied to improve this aspect, by enabling therapeutic agents to remain in circulation for longer periods [128-130] or by directing them towards specific sites [67, 101, 131]. A similar approach can be made to protect AA in the bloodstream from degradation or metabolization and even to increase the amount that reaches the mammary gland.

The described potentialities of nanotechnology in the field of dairy cow nutrition are merely an example of what can be achieved among countless possibilities but are more than enough to highlight how a multidisciplinary approach could improve the feeding of these animals.

In this work, nanotechnology was applied to the selective AA supplementation in dairy cows as an alternative rumen-protection product. To do so, the selected nanoparticles (NP) would have to fulfill the following requirements:

- Resist the microbial digestion that occurs in the rumen and protect their AA content across this;
- Release their AA cargo in the small intestine (so it can be absorbed into the bloodstream) or in the blood (should the NP themselves be absorbed at intestinal level);
- Have the capability of large-scale production in a cost-effective manner and of resisting the usual conditions of industrial feed production.

The proposed approach takes advantage of rumen resistant substances and applies them using nanotechnology to attempt to develop an effective RP delivery system capable of effectively protecting Lys and supplying to the blood of dairy cows where it can be used for milk production.

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Rationale of the Thesis

When considering high production animals, the main objective of dairy cow nutrition is to improve the animal's production and efficiency. In the recent years, and coupled with environmental concerns, the efficiency of nitrogen conversion from feed to milk has increasingly gained importance and is seen as one of the most lucrative aspects of milk farming. With the discovery of which amino acids were limiting production it became possible, at least in theory, to improve production while reducing the cost of the feeds, by ensuring an adequate amino acid (AA) profile in the animal, as described previously.

It has been found that lysine (Lys) is one of the first limiting AA, due to it being deficient in the usual corn-based diets coupled with its reduced intestinal uptake. However, due to the dairy cows' more complex digestive tract, the supplementing of this specific AA remains a dauting task, with the available rumen-protection options on the market not providing a consistent and effective response to current needs.

In this work, an alternative approach towards a delivery of Lys into the bloodstream, relying on nanotechnology, is proposed to attempt to overcome the limitations of the existing rumen-protection technologies. This approach relies in components that are naturally resistant to ruminal digestion to produce nanosized Lys carriers, known to have increased ruminal clearance and intestinal uptake due to their physical properties. To achieve this, two main objectives were defined:

- Development of NP capable of transporting and protecting Lys from digestion in the rumen, being able of a scalable and cost-effective production as well as to resist the industrial processing of animal feed products;
- Validation, through in *vitro*, *ex vivo* and *in vivo* assays, of the developed NP's ability to resist digestion in the entire bovine digestive tract and deliver their Lys content into the bloodstream.

With these two main objectives in mind, the work was performed and directed towards not only to develop a functional AA rumen-protection and delivery system but also to assess whether the said system could be produced at an industrial level, thus potentially becoming a product to be used in dairy nutrition.

Chapter 1. Lysine quantification method

Context

As previously stated, the main objective of this project is to increase lysine (Lys) supply through nanoparticle (NP) formulation to overcome the deficit of raw ingredients in this amino acid, thus ensuring the requirements of dairy cows for it. To guarantee a consistent delivery, it is essential to be able to accurately quantify the amount of Lys that can be loaded in the NP, as well as the amount that is lost during production or that can be released over time. To achieve this, a high-performance liquid chromatography (HPLC)-based quantification method, with fluorescence detection, was adapted from the literature and validated, to quantify the amount of Lys encapsulated in the NP. To ensure that this method was applicable within the expected media conditions, it was previously validated in three different media: 1) Li_2CO_3 buffer (chosen as the standard reaction media for the derivatization reaction of the amino acid (AA) [1]), 2) phosphate buffer solution (PBS) (to mimic cellular assay conditions) and 3) rumen inoculum (to quantify Lys after ruminal incubation).

1.1. Method development

1.1.1. Materials

Amino acid standard solution (AA-Mix), L-lysine monohydrochloride, lithium carbonate, chloroform, dansyl chloride, dimethyl sulfoxide (DMSO), methylamine hydrochloride, triethylamine and sodium acetate were purchased from Sigma-Aldrich (St. Luis, MO, USA). L-phenylalanine ethyl-ester hydrochloride, used as internal standard (IS), was obtained from Fluka (Fluka Chemie GmbH, Buchs, Switzerland), acetic acid from VWR Chemicals (VWR International S.A.S., Fontenay-sous-Bois, France), and both acetonitrile and methanol from Honeywell (Honeywell Riedel-de Häen AG, Seelze, Germany). Dulbecco's Phosphate Buffered Saline (PBS) 10x was purchased from Gibco ® (Invitrogen Corporation, Paisley, UK). Aqueous solutions were prepared with double-deionized water (Arium Pro, Sartorius AG, Göttingen, Germany).

1.1.2. Sample preparation

Considering that Lys is a non-chromogenic substance, dansyl chloride was chosen as the derivatization agent, as it is widely used in the quantification of AA [2, 3]. In short, 100 µL of dansyl solution (9.8 mM in acetonitrile) were added to 100 µL of sample or standard solution, supplemented with an accurate volume of the IS solution to achieve 100 µM, homogenized in the vortex and incubated for 30 min at 60 ˚C, protected from the light, to allow the derivatization reaction to occur. Afterwards, the resulting solutions were added to 10 µL of methylamine chloride (0.2 M), and homogenized in the vortex, to consume the excess of derivatizing agent that did not react. The final solution was then centrifuged and transferred to a 200 µL glass insert, placed in a 2 mL injection vial and placed in the autosampler.

1.1.3. Chromatographic conditions

The following conditions were selected after an optimization process and were used throughout this entire work.

The HPLC equipment was composed by two Jasco PU-2080 Plus Intelligent HPLC Pumps (Japan) and an automatic sample injector (Jasco AS-2057 Plus Intelligent Sampler), with a Jasco FP-920 Intelligent fluorescence detector programmed with 330 nm for the excitation and 508 nm for the emission wavelengths. The used column was a Kinetex EVO C18 100 Å (100.0 x 3.0 mm, 2.6 µm). The aqueous phase (A) was composed of sodium acetate at 0.02 M with triethylamine at 0.02%, pH set to 4.5 with acetic acid, while the organic phase (B) was a 1:9 mixture of A and methanol, respectively. The flow rate was 400 µL/min and the injection volume was 20 µL. The total run length was 22 min under the following gradient – 0 min: 47% (A), 13 min: 14% (A), 15 min: 14% (A), 17 min: 47% (A), 22 min: 47% (A).

Using this method, dansylated Lys-retention time was 13.3 min while the internal standard (IS) peak appeared at 15.8 min. A calibration curve was constructed using standard solutions of L-Lys plus IS with the final Lys-concentrations of 200, 100, 50, 25, 12.5, 6.25, 1 and 0 µM.

1.2. Method optimization and validation

1.2.1. Method optimization

The chromatographic conditions of this method were initially tested using an AA-mix (containing 16 L-AA, among which L-Lys) to determine if it was effective in separating the different AA, with special focus on ensuring that no other peak was interfering with that of Lys.

The derivatization process was very similar to the method described in the literature [1], with the only exception of a 10-fold increase in the dansyl chloride solution's concentration, to grant sufficiency to fully derivatize complex matrices and concentrated samples.

As to the chromatographic conditions, the initial gradient [1] was altered in order to reduce total run length, without compromising the separation of Lys from the remaining components. Since in the initial method [1] Lys only eluted from the column with higher percentages of the organic phase, the run length reduction was achieved by increasing the amount of this phase in the initial minutes of the run and only slowing this increase at higher values, to ensure that Lys peak would still be properly separated from the neighboring ones. Different organic phases (acetonitrile, methanol and a 1:1 mixture) were also tested to improve the peak separation and methanol was found to be the most suitable for this method. Several combinations of gradients with the different organic phases were considered and evaluated, with the following being found to be the best combination: 0 min: 47% (A), 13 min: 14% (A), 15 min: 14% (A), 17 min: 47% (A), 22 min: 47%. This gradient rendered a much shorter run length than the original method [1], without compromising the effective separation of Lys from the other components.

Norvaline, norleucine, p-chlorophenilalanine, L-Lys ethyl-ester and L-phenylalanine ethyl-ester were considered as potential IS to improve the method, being considered for their chemical similarity and peak's proximity to that of Lys but without any overlap with it. With the considered conditions, L-phenylalanine ethyl-ester presented a peak closer to the one of L-Lys, without

overlapping with it or with any other peak in the AA-mix, thus it was selected as the IS for this method.

1.2.2. Validation samples' preparation

For the method validation, three replicates of calibration curves were prepared in each of the 3 matrices, constructed using standard solutions of L-Lys plus IS with the final Lys-concentrations of 200, 100, 50, 25, 12.5, 6.25, 1 and 0 µM. Replicates of the intermediate concentration of 25 µM, were also used as chromatographic control (CC) in some of the validation parameters.

The aqueous Li_2C0_3 buffer was prepared at 0.04 M with pH adjusted to 9.5 using acetic acid. PBS buffer was prepared by diluting PBSin water, as described by the manufacturers (pH adjusted to 7.4). Rumen inoculum was freshly collected from fistulated cows and filtrated using 4 layers of linen cloth [4]. All three matrices were diluted 1:1 in Li_2CO_3 buffer (0.04 M, pH 9.5), prior to derivatization.

All samples were derivatized with dansyl chloride prior to analysis, as previously mentioned. All replicates were individually and separately prepared, derivatized and stored until further analysis.

1.2.3. Validation parameters

To properly characterize and validate the quantification method, several parameters were determined and evaluated: 1) selectivity, 2) linearity, 3) limit of detection (LOD), 4) limit of quantification (LOQ), 5) precision, 6) accuracy and 7) sample stability.

Selectivity is the methods' capability to differentiate the analyte from the remaining components of the matrix [5]. As aforementioned, the HPLC gradient was previously optimized to achieve a complete baseline separation between dansylated Lys and the IS, as well as from potentially interfering compounds from the diverse matrices under study. Using this gradient, no other peaks were observed near both Lys and IS retention times, indicating

that the method allowed an effective separation of the components in all three matrices (Figure 1).

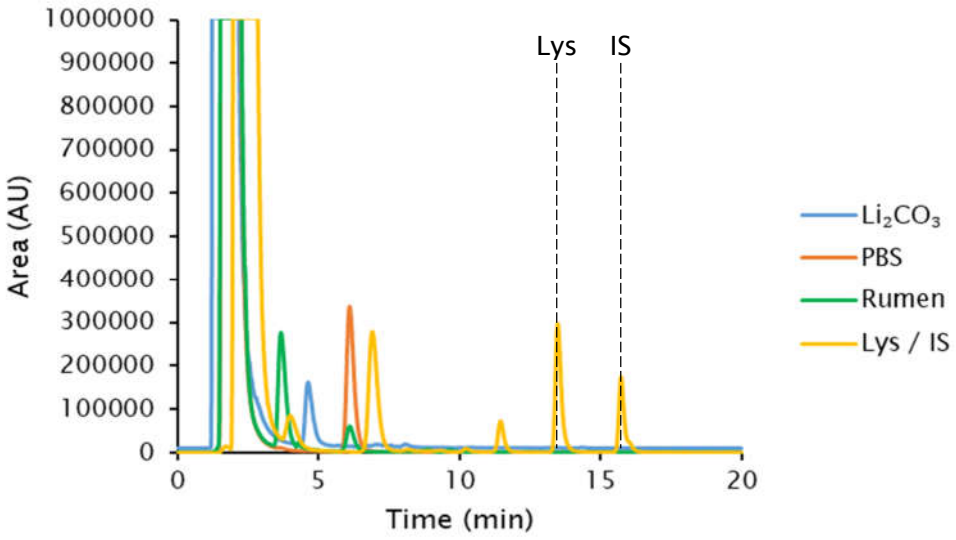

Figure 1 – Chromatogram of Li_2CO_3 buffer, phosphate buffer solution (PBS), rumen inoculum and lysine (Lys) with internal standard (IS) in Li_2CO_3 buffer.

Linearity was evaluated by plotting calibration curves, constructed as previously described, using each media as basis. A good linearity ($R > 0.9995$) was obtained, for all three matrices. The plotted regression curves for Li_2CO_3 buffer, PBS and rumen inoculum are presented in Table 1. These curves rendered linear and reproductible equations in the tested concentration range (1-225 µM for Li_2CO_3 and PBS, and 5-225 µM for rumen inoculum). It should be noted that the range for which the rumen inoculum curve was valid was slightly smaller, corresponding to the expected concentrations to be found under *in vivo* assays.

The **LOD** and **LOQ** were determined using the signal-to-noise method for all three matrices (Table 1). The obtained values were very similar for both buffers but slightly higher for rumen inoculum, as a result of a more complex organic matrix (as can be perceived from the blank chromatograms in Figure 1).

Table 1 – Regression analysis of calibration curves, detection limit (LOD) and quantification limit (LOQ) in Li_2CO_3 buffer, phosphate buffer solution (PBS) and rumen inoculum (n=3).

Matrix	Range (µM)	Slope	Intercept	Correlation Coefficient	LOD (µM)	LOQ (µM)
Li_2CO_3	1-225	0.011265	-0.009604	0.9999	0.12	0.41
PBS	1-225	0.009982	0.007488	0.9998	0.16	0.53
Rumen	5-225	0.031104	0.045309	0.9996	1.24	4.14

Precision and **accuracy** were determined (equation 1 and 2, respectively) by analyzing six replicates of a CC sample (25 µM Lys in each media) over 3 different days. Intra- and inter-day relative standard deviation (RSD) were also calculated using these samples (Table 2).

$$\text{Precision} = \frac{\text{CC sample standard deviation}}{\text{CC sample mean}} \times 100(\%) \quad (1)$$

$$\text{Accuracy} = \frac{\text{CC sample mean concentration}}{\text{CC sample actual concentration}} \times 100(\%) \quad (2)$$

In both cases, the samples were derivatized immediately before the analysis. Precision depicts how close the values of different replicas are to each other, whereas the accuracy of an analytical method relates to how close the rendered values are to the nominal value. Based on the results achieved (Table 2), all values are within the acceptable ranges according to [6], except for the inter-day RSD for the PBS matrix, indicating that the method is adequate for the quantification of Lys, but some variations could occur in samples prepared in PBS due to external effects.

Table 2 – Determined values of accuracy, precision and intra-day and inter-day relative standard deviation (RSD) values for Li_2CO_3 buffer, phosphate buffer solution (PBS) and rumen inoculum, n=6.

Matrix	Concentration (µM)	Accuracy (%)	Precision (%)	RSD (%)	
				Intra-day	Inter-day
Li_2CO_3	25	93.7%	2.9%	1.8%	12.6%
PBS	25	92.3%	2.4%	2.1%	22.1%
Rumen	25	91.8%	10.3%	8.9%	8.5%

Sample stability was estimated for both derivatized and non-derivatized CC samples, over the course of 96 h in three different storage conditions: room temperature (RT), 4 ˚C and -20 ˚C (Table 3, A and B). The results showed that non-derivatized samples were not stable under any of the tested storage conditions. The same can be said for the derivatized samples stored at -20˚C. This instability was mainly due to the degradation of the IS, and to a smaller extent to the degradation of Lys, as it was observed by a considerable decrease in both peak values. However, the derivatized samples in PBS and rumen inoculum appear to be stable for up to 96 h if stored at RT or 4 ˚C, whereas derivatized samples in Li_2CO_3 only appear to be stable for 24 h under these storage conditions.

To address the apparent reduced stability of the IS in the samples, their storage prior to the addition of IS was considered, with the latter being prepared daily and only added to the samples after their storage and before the derivatization, which was only performed immediately before injection (Table 3, C). This method of sample storage greatly increased the stability of the samples in every storage condition, which began to show some instability only after 94 h of storage, regardless of the storage condition.

Considering the obtained results (Table 3), it is suggested that this method should ideally be applied in the quantification of analytes that are able to retain their stability for up to 48 h of storage and that would be analyzed within this period of time, but also that the IS should be prepared and added, prior to

derivatization, as close as possible to the injection of the samples. Samples could be stored for up to an additional 48 h prior to processing and analysis, but, if possible, this should be avoided to ensure that they do not suffer any kind of degradation or alteration.

Table 3 – Stability results for derivatized (A), non-derivatized (B) and internal standard (IS) added immediately prior to derivatization (C) samples, at 24 h, 48 h and 96 h of storage in Room Temperature (RT), 4 ˚C and -20 ˚C for Li_2CO_3 buffer, phosphate buffer solution (PBS) and rumen inoculum, n=6. All values shown in percentage (%) and represent the relative standard deviation when compared to the initial samples.

Matrix	**24 h**			**48 h**			**96 h**		
	A	B	C	A	B	C	A	B	C
Stored at RT									
Li_2CO_3	99	286	102	75	343	109	76	151	83
PBS	100	211	100	101	588	105	100	1976	85
Rumen	100	8414	99	107	5375	100	106	2105	95
Stored at 4 ˚C									
Li_2CO_3	101	167	103	115	111	108	344	155	83
PBS	100	115	100	101	168	105	100	189	85
Rumen	99	69	99	101	83	101	99	4891	96
Stored at -20 ˚C									
Li_2CO_3	338	134	102	400	85	108	368	91	83
PBS	105	105	100	524	122	106	410	110	85
Rumen	474	147	99	287	140	101	522	167	96

Conclusions

Much effort was spent in developing an effective method to quantify Lys in several media to be afterwards applied in the quantification of this AA. This is extremely important for the overall objective, since the amount of Lys that is loaded in the NP is directly related to the amount of Lys that would be possible to supply to the animal and the amount of Lys that would be wasted in the production. The results presented in the current section indicate that the method is indeed adequate for the quantification of Lys and can be successfully applied in any of the tested media: Li_2CO_3 and PBS buffers or rumen inoculum. This method was applied throughout this entire project in the quantification of Lys.

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Chapter 2. Nanoparticle production

Context

The main objective of this work was the development of nanoparticles (NP) capable of resisting digestion in the bovine digestive tract, particularly in the rumen, and supply the animal with lysine (Lys). In order to achieve this, the NP should be able to fulfill the following requirements:

- Be efficiently loaded with Lys;
- Be capable of resisting ruminal digestion, thus protecting their Lys content;
- Be able to be produced in a scalable and cost-effective manner;
- Be capable of resisting the conditions of the industrial processes that animal feeds undergo during production, particularly the high temperatures achieved;
- Possess an adequate shelf-life, enabling the product to be stored for a long time after purchase and not having to be used immediately after production.

Lipids, in particular saturated fatty acids, were chosen as the NP building blocks, due to their high resistance to ruminal digestion and to lipid NP's simple and easily scalable production method. The fact that these lipids are generally regarded as safe (GRAS) and that they are not very expensive were also relevant when considering them for this application. This section focuses on the production of lipid NP and their characterization to assess their properties and evaluate if they would be suitable for the overall objective of the work. An optimization of the NP production parameters was also performed to maximize the amount of Lys that they could incorporate.

2.1. Nanoparticle production and characterization methods

2.1.1. Materials

Lab grade stearic acid and SPAN® 80 were purchased from Merck (© Merck KGaA, Darmstadt, Germany) and pharmaceutical excipient grade stearic acid and Tween® 60 were obtained from Acofarma (® Acofarma – Acofarma Distribución, S.A. BCN, Spain). Arachidic acid, Lab grade Tween® 60, Tween® 80, poly(vinyl alcohol), L-lysine monohydrochloride, lithium carbonate, dansyl chloride, methylamine hydrochloride, triethylamine and sodium acetate were purchased from Sigma-Aldrich (St. Luis, MO, USA), Miglyol 812 from Caelo (Caesar & Loretz GmbH, Hilden, Germany), L-phenylalanine ethyl-ester hydrochloride from Fluka (Fluka Chemie GmbH, Buchs, Switzerland), acetic acid from VWR Chemicals (VWR International S.A.S., Fontenay-sous-Bois, France), and acetonitrile and methanol from Honeywell (Honeywell Riedel-de Häen AG, Seelze, Germany). Precirol ATO 5 was kindly provided by Gattefossé (Saint Priest Cedex, France). Aqueous solutions were prepared with double-deionized water (Arium Pro, Sartorius AG, Göttingen, Germany).

2.1.2. Sonication-based nanoparticle production

Three types of lipid NP were produced and considered in this work: solid lipid nanoparticles (SLN) [1], nanostructured lipid carriers (NLC) [2] and multiple-lipid nanoparticles (MLN) [3]. Combinations of lipids, surfactants and NP types tested for NP production are presented in Table 1.

Table 1 – Combinations of solid lipids, liquid lipids and surfactants tested for different nanoparticle (NP) production.

NP[1]	Solid lipid	Liquid lipid	Surfactant
SLN	Stearic acid	----------	Tween® 60
SLN	Stearic acid	----------	Tween® 80
SLN	Stearic acid	----------	poly(vinyl alcohol)
SLN	Arachidic acid	----------	Tween® 60
SLN	Arachidic acid	----------	Tween® 80
SLN	Arachidic acid	----------	poly(vinyl alcohol)
NLC	Stearic acid	Miglyol 812	Tween® 60
NLC	Arachidic acid	Miglyol 812	Tween® 60
MLN	Stearic acid	Miglyol 812	SPAN® 80 + Tween® 60
MLN	Arachidic acid	Miglyol 812	SPAN® 80 + Tween® 60

[1]Solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and multiple lipid nanoparticles (MLN).

Regarding SLN and NLC, lipids and surfactants were melted in a water bath with the temperature set at 5 ºC above their melting point. A solution of Lys, prepared in ultra-pure water, using L-Lys monohydrochloride, was heated at the same temperature and added to the melted lipids. The resulting emulsion was sonicated using a probe-type sonicator for 5 min at 80% amplitude (model VCX-130 with a VC 18 probe, Sonic & Materials Inc., Newtown, CT, USA) to form a nanoemulsion and afterwards allowed to cool down at room temperature (RT), rendering an aqueous suspension of NP, which was stored at RT until further use. Small variations of this method were also considered: adding the surfactant in the aqueous phase instead of with the lipids and of cooling down the final emulsion at 4 ºC rather than at RT, to address Lys loading issues.

For the MLN, the production method was slightly different [3]. After a first sonication for 14 min at 70% amplitude, the formulations were allowed to cool for 10 min at RT and afterwards a solution, containing a second surfactant, was added and the emulsion sonicated once more, this time for 8 min at 60% amplitude, before allowing NP formation.

2.1.3. Water-in-oil-in-water double emulsion

Lipids were dissolved in chloroform and a small volume of a Lys aqueous solution added to this organic phase. The resulting emulsion was sonicated using a probe-type sonicator for 2 min at 70% amplitude (model VCX-130 with a VC 18 probe, Sonic & Materials Inc., Newtown, CT, USA). An aqueous solution of Tween® 60 was afterwards added to the nanoemulsion and then stirred at RT for 3 h to enable chloroform evaporation. The final NP suspension was then stored at RT until further usage. Only SLN were produced using this water-in-oil-in-water (W/O/W) method [4].

2.1.4. High-pressure homogenization-based nanoparticle production

Lipids and surfactants were melted in a water bath with the temperature set at 5 ºC above their melting point. A solution of Lys, prepared in ultra-pure water, using L-Lys monohydrochloride, was heated at the same temperature and added to the melted lipids. The resulting emulsion was pre-homogenized (T25 digital, ULTRA TURRAX ® - IKA®-Werke GmbH & Co. KG, Staufen, Germany) and afterwards homogenized at 5.5 Bar and 80 ºC (SPCH-1080 EP Ultra High-Pressure Homogenizer, Stansted Fluid Power Products Ltd, Stansted, UK). The resulting nanoemulsions were collected and either placed in the homogenizer for re-processing (additional cycle) or allowed to cool at RT to enable NP formation. After the adequate number of high-pressure homogenization (HPH) cycles were performed, the NP dispersion were placed in glass vials and stored at RT until further usage. This method was only used to produce SLN [5].

2.1.5. Dynamic light scattering

The NP formulations were characterized in terms of mean diameter and size distribution profile (polydispersity index – PdI) by dynamic light scattering (DLS) using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). A wavelength of 660 nm, a temperature of 20 ˚C, and a detection angle of 90˚ were used in the measurements and the refractive indexes were 1.59 and 1.33, for the NP and the solvent (water), respectively. All samples were diluted using ultra-pure water.

2.1.6. Electrophoretic light scattering

The NP dispersions were characterized in terms of zeta potential by electrophoretic light scattering (ELS) using a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). A wavelength of 660 nm, a temperature of 20 ˚C, and a detection angle of 90˚ were used in the measurements and the refractive indexes were 1.59 and 1.33, for the NP and the solvent (water), respectively. All samples were diluted using ultra-pure water.

2.1.7. Transmission electron microscopy

The NP formulations were previously diluted 100x in ultra-pure water. Samples were prepared by dropping 10 µL of NP dispersion on a copper-mesh grid, the excess being removed using filter paper. Uranyl acetate was used as contrasting agent (10 µL of a 0.75% solution were added to the grid) and the excess was removed using filter paper. The grids were observed using a JEM-1400 Transmission Electron Microscope (TEM) (JEOL Ltd., Tokyo, Japan), with an accelerating voltage of 80 kV.

2.1.8. Lysine encapsulation efficiency

The Lys encapsulation efficiency (%Lys) was determined by a direct and an indirect method, both of which require a previous separation of the Lys that was encapsulated within the NP and the Lys that might not have been encapsulated (free Lys). In the direct method, encapsulated Lys was quantified and compared with the amount of Lys initially added (equation 1), whereas in the indirect method, free Lys was quantified and compared with the amount of Lys initially added to determine the %Lys (equation 2). The percentage of Lys in the NP composition, Lys loading capacity (%LC), was also calculated (equation 3).

$$\%\text{Lys}\ (\%) = \frac{\text{Amount of Lys in the NP}}{\text{Total amount of Lys}} \times 100 \qquad (1)$$

$$\%\text{Lys}\ (\%) = \frac{\text{Total amount of Lys - amount of Lys in the supernatant}}{\text{Total amount of Lys}} \times 100 \quad (2)$$

$$\%\text{LC}\ (\%) = \frac{\text{Amount of encapsulated Lys}}{\text{Amount of Lipid + Amount of Surfactant}} \times 100 \quad (3)$$

The NP suspensions were firstly diluted 100x in ultra-pure water and filtered with Amicon ® Ultra Centrifugal Filters Ultracell-50 kDa (EMD Millipore, Darmstadt, Germany) at 2200 x*g* for 33 min using an Allegra® X-15R centrifuge (Beckman Coulter; Pasadena, CA, USA). The supernatant, containing the free non-encapsulated Lys, and the pellet, after removal from the filters, were both collected for Lys quantification, utilizing the previous developed high-performance liquid chromatography (HPLC) method, described in **Chapter 1**. The Lys in the supernatant was quantified and %Lys determined via the indirect method. The pellets were destroyed, to release their Lys content that was quantified and %Lys determined via the direct method.

2.1.9. Ruminal resistance assay

Ruminal contents were obtained from three multiparous (number of parity = 2) adult Holstein cows, dry and not pregnant that were fitted with a ruminal cannula (10 cm diameter; Bar Diamond Inc., Parma, ID, previously approved and licensed by the Portuguese National Authority for Animal Health, *Direção-Geral de Alimentação e Veterinária*, permit 0421/000/000/2015). The cows were housed at the Vairão Agricultural Campus of Abel Salazar Biomedical Sciences Institute, University of Porto (Vila do Conde, Portugal). Cows were handled in strict accordance with good animal practice as defined by the national authority and European Union Directive 2010/63/EU. Experimental animal procedures were approved by the Local Animal Ethics Committee of Abel Salazar Biomedical Sciences Institute, University of Porto, licensed by the Portuguese Directorate-General of Food and Veterinary Medicine of the Ministry for Agriculture and Sea, and conducted by trained scientists (FELASA category C). Each cow was fed a total mixed ration, based on corn silage or haylage, and had fresh drinking water available at all times. Ruminal contents

were collected before the morning meal from the 4 quadrants of the rumen and placed in a 4 L pre-warmed (39 °C) thermal jug. In the laboratory, the ruminal digesta of each cow was homogenized and strained through 4 layers of linen cloth at 39 °C in O_2-free CO_2 atmosphere. The interval between the collection of the ruminal contents and incubation never exceeded 60 min. One part of the strained ruminal fluid, pH 6.3, was diluted anaerobically into 4 parts of Kansas State Buffer [6] and mixed at 39 °C in an O_2-free CO_2 atmosphere. Thirty milliliters (25 mL of buffered ruminal fluid and 5 mL of each NP formulation) were dispensed anaerobically into 125 mL serum bottles (Sigma-Aldrich Inc., St. Louis, MO, USA) containing 250 mg (dry matter) of wheat straw, sealed with butyl rubber stoppers and aluminum crimp caps (Sigma-Aldrich Inc., St. Louis, MO, USA), and incubated in a water bath at 39 °C. Each NP formulation, as well as blanks containing no NP, were incubated in quadruplicate. Two of these replicates of each sample and blanks were placed in an ice bath immediately after the addition of the NP formulation to serve as negative controls (T0 samples, where T represents the time and 0 indicates that no incubation occurred). Fermentations were stopped after 24 h by cooling the bottles in an ice-slurry bath (Tf samples, where T represents the time and f indicates that the incubation occurred until the end of the designated time period). The samples were subsequently compared with NP that did not contact the rumen inoculum (Ti samples, where T represents time and i represents that the NP where characterized after production).

The contents of the flasks were transferred to Falcon tubes and centrifuged at 500 x*g* for 5 min to separate any remaining feed and the heaviest microorganisms, such as protozoa, from the NP. The supernatant was collected and centrifuged at 30000 x*g* for 20 min to separate the NP from the bacteria and other microorganisms that did not deposit in the first centrifugation. Both supernatants and deposits were collected for characterization in terms of size, PdI, zeta potential and morphology. This procedure was also performed on the rumen inoculum and buffer mixture without the addition of any NP formulation (blanks), and these results were used as controls to verify the separation of the NP and rumen inoculum.

2.1.10. Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed using a PerkinElmer Pyris 1 DSC 200 F3 Maia® (Netzschm, MA, USA). Tested lipids, Lys, their physical mixture (mixture of Lys and lipids without NP production) and NP produced with these lipids were weighed directly on aluminum pans and an empty pan was used as a reference. All samples were scanned between 20 ˚C and 90 ˚C, with a heat rate of 10 ˚C per min and cooled down at 40 ˚C per min. The values of onset (˚C), melting point (˚C) and enthalpy (ΔH) were calculated from the data using Origin(Pro) (OriginLab Corporation, Northampton, MA, USA), whereas the recrystallization index (RI) was calculated using the equation 4 [7].

$$RI(\%) = \frac{\Delta H \text{ NP } (J/g)}{\Delta H \text{ bulk material } (J/g) \times \text{Concentration lipid phase } (\%)} \times 100 \qquad (4)$$

2.1.11. Box-Behnken design-based optimization

Box-Behnken designs (BBD), mathematical models used for parameter optimization with a reduced number of required runs [8, 9], were applied to optimize NP production parameters with the goal of maximizing %Lys and %LC. The independent variables in the optimization were the amount of lipid (X1), the amount of surfactant (X2) and the amount of Lys (X3), while the dependent variables were the %Lys (Y1) and %LC (Y2) (Table 2). Desirability analysis was performed in order to maximize both %Lys and %LC with analysis of variance (ANOVA) using STATISTICA 12 software (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP.).

Table 2 - Box-Behnken design variables and levels.

		Level		
Independent Variables		-1	0	+1
X_1	Lipid amount (mg)	400	500	600
X_2	Tween® 60 amount (mg)	75	100	125
X_3	Lys amount (mg/mL)	4.45	5.68	6.81
Dependent Variables				
Y_1	Encapsulation efficiency (%)			
Y_2	Loading efficiency (%)			

2.1.12. Shelf-life stability

To evaluate the shelf-life of the developed NP formulations (the amount of time they can be stored without compromising their properties), they were stored at RT and assessed, after 1, 2 and 4 weeks, in terms of their size, size distribution profile, zeta potential and %Lys, using the previously described methods.

2.1.13. Nanoparticle lyophilization

Firstly, 2 mL of NP dispersion, supplemented with 1% AEROSIL®, were frozen overnight at -80 ˚C (Deep Freezer GFL®, Burgwedel, Germany) and afterwards were lyophilized using a laboratory freeze drier (LyoQuest -85 plus v.407, Telstar ® Life Science Solutions, Terrassa, Spain) for 72 h at -80 ˚C under a pressure of 0.4 mbar. The lyophilized NP were resuspended in the same volume of water (2 mL) and characterized in terms of size, PdI and zeta potential as previously described.

2.1.14. Statistical analysis

All statistical analyses were performed with IBM SPSS Statistics (SPSS 25.0, Armonk, NY, USA). ANOVA was performed to compare multiple groups of independent samples. When the effect was statistically different ($P \leq 0.05$), the differences between the respective groups were compared with a post-hoc test (Pairwise, $P \leq 0.05$).

2.2. Component selection

In order to select the components with which to develop the optimal NP formulation, all proposed NP were characterized in terms of their dimensions, surface charge, ability to carry Lys and ruminal resistance. The ability to carry Lys and ruminal resistance were considered preferential regarding the remainder as they are essential to achieve the overall objective of the present work.

2.2.1 Size, polydispersity index and zeta potential

Saturated fatty acids were considered for NP production due to their ruminal resistance, and, among them, stearic and arachidic acids were chosen for their high melting points [10]. This aspect is relevant as manufacturing animal compound feeds frequently involves high temperatures. It was possible to produce NP formulations with all the proposed lipid and surfactant combinations (detailed in Table 1), except when using poly(vinyl) alcohol where the formulations solidified and were thus discarded. Table 3 presents the mean diameter, PdI and zeta potential values obtained for all produced formulations.

The formulations rendered NP (Table 3) with sizes ranging from 130 nm to 450 nm and with highly negative zeta potential values (lower than -30 mV). Considering the NP size, no statistically significant differences were found among all SLN produced with Tween® 60 and between them and the NLC, being MLN significantly different from all other NP produced. For SLN, the use of

Tween® 80 resulted in NP with smaller sizes than Tween® 60 and these results are in accordance to the literature [11]. All formulations showed PdI values between 0.12 and 0.25, indicative of monodispersed NP size populations, except for MLN that possessed significantly higher polydispersity values (averaging 0.4), indicative of a wider range of NP sizes [12]. Regarding zeta potential, all formulations showed highly negative surface charges, averaging from -30 to -40 mV. These negative values were expected since both lipids and surfactants present negative charges. MLN presented an even lower potential charge (-60 mV), the only ones significantly different from the remainder NP. This is probably due to the presence of large amounts of SPAN® 80 that usually present a lower zeta potential than Tweens® [13]. These high values in modulus imply that NP will be less likely to aggregate and thus remain more stable as a dispersion [14

Table 3 – Mean diameter, polydispersity index (PdI) and zeta potential values for all tested nanoparticles (NP) composed of stearic (S) or arachidic (A) acids, n = 3.

NP[1]	SLN S *	SLN S #	SLN A *	SLN A #	NLC S *	NLC A *	MLN S *	MLN A *	SEM[2]	P value
Size (nm)	451[a]	303[b]	402[a]	162[b]	312[a]	412[a]	215[b]	208[b]	26	<0.001
PdI	0.25[a]	0.15[a]	0.20[a]	0.13[a]	0.17[a]	0.22[a]	0.41[b]	0.39[b]	0.02	<0.001
Zeta (mV)	-38[a]	-31[a]	-37[a]	-34[a]	-39[a]	-46[a]	-61[b]	-59[b]	2	<0.001

[1]Solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and multiple lipid nanoparticles (MLN);

[2]Standard error of the mean;

*Made with Tween® 60 surfactant;

[#]Made with Tween® 80 surfactant;

In the same row, means with different superscript letters are statistically different ($P \leq 0.05$).

2.2.2. Lysine encapsulation efficiency

Both a direct method (quantification of loaded Lys) and an indirect method (quantification of the unloaded Lys) were used to determine the %Lys of the NP. However, with the direct method, %Lys values were very variable and highly inconsistent, thus not considered in this work, whereas the indirect method rendered consistent and reproduceable %Lys values.

In fact, a considerable number of different attempts were performed to determine %Lys via the direct method. These attempts included several organic and inorganic solvents, such as acetonitrile, chloroform, dimethyl sulfoxide, ethanol, isopropanol, methanol and n-hexane, with and without ultrasound aid, increased temperatures or both, to try and destroy the NP, in order to release the encapsulated Lys so that it could be quantified. Many of the treatment combinations were not able to properly destroy or dissolve the NP and the attempts that succeeded in this destruction were not able to effectively and consistently release the loaded Lys, rendering inconsistent %Lys values. These inconsistencies sometimes rendered variations between replicas of up to 70% of %Lys, implying up to 10-fold differences in the amount of Lys that was quantified. The non-reproducibility of the direct determination of %Lys was attributed to an ineffective separation of the Lys from the lipids. To attempt to surpass this obstacle, several lipid separation processes were performed, relying in different solvents/solvent mixtures, such as acetonitrile:fumaric acid:cyclohexane or variations with previously described solvents, and liquid-liquid extraction. Raising the pH above the isoelectric point of the Lys, so that it would be negatively charged and repel the lipids (that also present a negative charge) was also considered. However, none of these approaches, or any combination of them, rendered consistent and reproduceable results.

The results regarding %Lys, with determination via indirect method, are presented in Table 4. MLN presented the highest %Lys (up to 90%), most probably due to the presence of large aqueous vacuoles within their lipid matrix and to the hydrophilic nature of Lys. Additionally, as these vacuoles are formed prior to the NP dispersion, all added Lys is expected to be trapped in

the internal vacuoles and not in the exterior aqueous phase that surrounds the MLN [3]. The NLC rendered lower %Lys values of up to 65%, when comparing with MLN, while the SLN reached only values of up to 40%. The higher values for the NLC, compared to the SLN, could be due to the presence of liquid lipid in the NP matrix that confers a less crystalline structure with more cavities and defects where the encapsulated molecules can be trapped [7, 15]. This structure can also reduce the effects of shifts in lipids (changes overtime that occur in lipidic structure that lead to a more stable polymorphic structure) that can cause the encapsulated molecules to be expelled [2]. SLN are composed of only solid lipids which render a more crystalline structure of hydrophobic molecules, resulting in reduced locations for molecule entrapment [15], thus a lower %Lys. No significant changes were found between the %Lys of SLN composed of arachidic or stearic acids with different surfactants (data not shown).

To summarize, results showed that %Lys was highest for MLN (80-90%), intermediate for NLC (approximately 60%) and lowest for SLN (approximately 40%). The %Lys varied significantly among the different types of NP but remained very similar within each type of NP, with no significant differences found, indicating that the lipid and surfactant did not have a great influence in the amount of Lys that could be loaded but that the structure of the NP was very influent.

It can be seen (Table 4) that all these NP are loaded with less Lys than the commercially available rumen-protected Lys products, which supposedly present no less than 180 mg of Lys per g of product [16-18]. However, studies that have been performed with such products [17, 19-24] report inconsistent and sometimes even negative impacts on milk production. The ineffectiveness of these products is most commonly due to poor ruminal protection or their infectivity in delivering Lys to the bloodstream [25, 26]. Nanotechnology was applied in this work to attempt to overcome these limitations. If an increased protection, an improved Lys intestinal absorption or a combination of these two phenomena could be achieved, it would be possible to improve results even with a reduced Lys-loading.

Table 4 – Lysine encapsulation efficiency (%Lys) for all tested nanoparticles (NP) composed of arachidic (A) or stearic (S) acid with Tween® 60 as surfactant and amount of Lys (mg) per amount of NP (g) n = 3.

NP[1]	SLN S	SLN A	NLC S	NLC A	MLN S	MLN A	SEM[2]	P value
%Lys (%)	39[a]	38[a]	59[b]	63[b]	83[c]	91[c]	3	<0.001
mg of Lys/g of NP	16[a]	16[a]	27[b]	25[b]	39[c]	43[c]	2	<0.001

[1]Solid lipid nanoparticles (SLN), Nanostructured lipid carriers (NLC) and multiple lipid nanoparticles (MLN);

[2]Standard error of the mean (SEM);

In the same row, means with different superscript letters are statistically different ($P \leq 0.05$).

2.2.3. Ruminal resistance of the nanoparticles

NP formulations were evaluated after 0 h (T0 samples) and 24 h (Tf samples) incubation with rumen inoculum and were compared to the initial NP (Ti). Regarding the ruminal stability assay for SLN composed of arachidic or stearic acids with Tween® 60 (Table 5), supernatants presented mean diameter values of 100-300 nm, similar to the blanks, while deposits had values of 300-500 nm, indicating that it was possible to separate the microorganisms from the NP, the latter being found in the deposit. Rumen inoculum without NP addition was used as control for both NP separation and microorganism sizes. The diameter values registered in the blanks were of microorganisms and not of NP, since they were not added in these samples. Additionally, it was observed that both T0 and Tf deposits presented similar sizes compared to the original formulations (Ti), suggesting that the NP could resist ruminal digestion. All other NP showed statistical differences between the initial values and those obtained after incubation in the rumen inoculum (Table 6), with some NP's size changing from 525 nm to 625 nm, in the case of arachidic acid SLN with Tween® 80, while others increasing from 200 nm to 550 nm, in the case of arachidic acid NLC. SLN composed of precirol ATO 5, a triglyceride with esters of palmitic and stearic acids, were also produced and used as a negative control as it is known that triglycerides rapidly degrade in the rumen [10, 27]. The results regarding these triglyceride-based NP (Table 6) were in accordance with the literature, as these SLN were not able to resist ruminal digestion.

Table 5 – Size and zeta potential results for solid lipid nanoparticles (SLN) composed of stearic and arachidic acids with Tween® 60 during the rumen stability assay. Results also shown for blanks and supernatants (SN) that contain bacteria (T0 and Tf) and deposits that contain SLN (Ti, T0 and Tf). Ti – SLN after production, T0 – SLN after 0 h of incubation and Tf –SLN after 24 h of incubation, n = 3.

SLN		Blank T0	Blank Tf	SN T0	SN Tf	SLN Ti	SLN T0	SLN Tf	SEM[1]	P value
Stearic acid with Tween® 60	Size (nm)	168[a]	77[a]	80[a]	211[a]	328[b]	445[b]	440[b]	37	<0.001
	Zeta potential (mV)	-18[a]	-12[a]	-23[a]	-17[a]	-37[b]	-49[c]	-49[c]	1	<0.001
Arachidic acid with Tween® 60	Size (nm)	155[a]	115[a]	195[a]	195[a]	455[b]	430[b]	430[b]	47	<0.001
	Zeta potential (mV)	-18[a]	-12[a]	-9[a]	-17[a]	-34[b]	-45[c]	-49[c]	1	<0.001

[1]Standard error of the mean (SEM)

In the same row, means with different superscript letters are statistically different ($P \leq 0.05$).

Table 6 – Size (nm) results for SLN composed of precirol ATO 5 with Tween® 60 or arachidic acid with Tween® 80, arachidic acid NLC and arachidic acid MLN during the rumen stability assay. Results for blanks and supernatants (SN) that contain bacteria (T0 and Tf) and deposits that contain nanoparticles (NP; Ti, T0 and Tf) are also shown. Ti – NP after production, T0 – NP after 0 h of incubation and Tf – NP after 24 h of incubation, n = 3.

NP[1]	Blank T0	Blank Tf	SN T0	SN Tf	NP Ti	NP T0	NP Tf	SEM[2]	P value
SLN - Precirol ATO 5 Tween® 60	$174^{a,b,d}$	$219^{a,b,d}$	132^{a}	$318^{b,e}$	487^{c}	$382^{c,e}$	$300^{b,d,e}$	53	0.005
SLN - Arachidic acid Tween® 80	151^{a}	106^{a}	49^{a}	332^{a}	120^{a}	226^{a}	480^{b}	93	0.063
NLC - Arachidic acid	70^{a}	128^{a}	368^{b}	$171^{a,c}$	$330^{b,c}$	368^{b}	467^{b}	54	0.001
MLN - Arachidic acid	70^{a}	$128^{a,b}$	135^{a}	90^{a}	151^{b}	168^{b}	352^{c}	24	<0.001

[1]Solid lipid nanoparticles (SLN), Nanostructured lipid carriers (NLC) and multiple lipid nanoparticles (MLN);

[2]Standard error of the mean (SEM);

In the same row, means with different superscript letters are statistically different ($P \leq 0.05$)

It was found that only SLN composed of stearic or arachidic acids and using Tween® 60 as a surfactant could resist ruminal digestion (Table 5). Indeed, the results obtained with DLS in this stability assay proved that the selection of components was adequate, since these NP were made of unbound stearic and arachidic acids that pass the rumen virtually unscathed [10, 28]. These two long chain fatty acids (C18 and C20, stearic and arachidic acid respectively) were particularly considered for this work since they are both saturated fatty acids with high melting points, and also due to the fact that stearic acid has the highest clearance among the usual lipids exiting the rumen [10]. On the contrary, NP made of triglycerides that were used as negative controls, that suffer some degree of digestion by the ruminal microbiota [10, 28], were not able to resist ruminal incubation (Table 6) as expected.

The SLN produced with Tween® 80 (Table 6) were found not to be as stable as SLN with Tween® 60 (Table 5). Both Tweens are composed of polyoxyethylene (20) sorbitan covalently bound to fatty acids. These fatty acids are stearic acid and oleic acid for Tween® 60 and Tween® 80, respectively. Stearic acid is a saturated fatty acid whereas oleic acid has one cis-double bound in the 9th carbon, both with 18 carbons [29]. This small difference significantly changes the hydrophobic portion of the molecule, increasing its steric hindrance and resulting in a more fluid and potentially less stable organization of the NP surface when using Tween® 80 [30]. Furthermore, studies have been made regarding the effects of these surfactants on microbial rumen fermentation and have found not to have any negative impacts [31, 32], or may even help to improve it [33].

The inability of NLC and MLN to resist ruminal degradation (Table 6) can be explained by the presence of liquid lipids in their lipid matrix, specifically miglyol® 812. The use of both liquid and solid lipids decreases the crystallinity of the lipid matrix and potentially increases substance entrapment while reducing expulsion due to lipid form shift [2]. However, it seems that ruminal conditions destabilize the liquid-solid lipid matrix, exposing it to microbial digestion, or that the liquid lipids themselves naturally suffer a more extensive degradation by the ruminal microbiota or even that a combination of these two

phenomena occurs, rendering these NP unable to successfully cross the rumen. The influence of the solid lipid to liquid lipid ratio was not assessed in the current work, since the objective was to compare the resistance of several NP to ruminal digestion and NLC and MLN were found to be extensively degraded in the rumen. This ratio could alter the ability of these NP to resist ruminal digestion, but the results suggest that in order for this to occur the amount of liquid lipid present would have to be so small that it would not be significant in any other of the considered properties [34], such as size and %Lys.

Regarding the zeta potential results (Table 5), the increases, in modulus, were expected since the rumen inoculum possess a negative potential value that increases the overall negative charge of the mixture. The differences between the values of the deposits and the supernatants, with the latter being similar to those of the blanks, also contributes to confirm that the separation protocol used was effective.

To summarize, the results obtained with DLS showed that only SLN composed of stearic or arachidic acids with Tween® 60 as surfactant (Table 5) were able to resist ruminal digestion, additionally maintaining a high zeta potential that favors their non-aggregation [35]. All other tested formulations showed clear signs of destruction or instability after 24 h of incubation with ruminal inoculum, the NP sizes at Tf were very different than the ones at the respective Ti and in some cases separation of the NP from the rumen inoculum was not possible (Table 6).

TEM photographs were taken to confirm NP resistance to ruminal digestion and to assess their morphology before and after incubation in the rumen. Figure 1 presents TEM images for SLN composed of stearic or arachidic acids with Tween® 60, the only NP that could resist the ruminal digestion, according to the previous DLS results. Both original NP (Ti samples) presented a spherical morphology with sizes similar to those observed with the DLS (ranging from 300 to 500 nm). After 0 h (T0 samples) and 24 h (Tf samples) of incubation with ruminal inoculum, NP maintained both their size and spherical shape. These results confirm the conclusions obtained with DLS analysis: that both

SLN composed of stearic or arachidic acids could resist digestion in the ruminal inoculum for up to 24 h of incubation. The observed NP sizes are adequate for intestinal uptake, even though they would most likely enter the bloodstream by phagocytosis through intestinal macrophages [36]. It has also been found that particles with sizes around 500 nm have an increased intestinal uptake [37], which would make the proposed formulations suitable for absorption at the intestinal level.

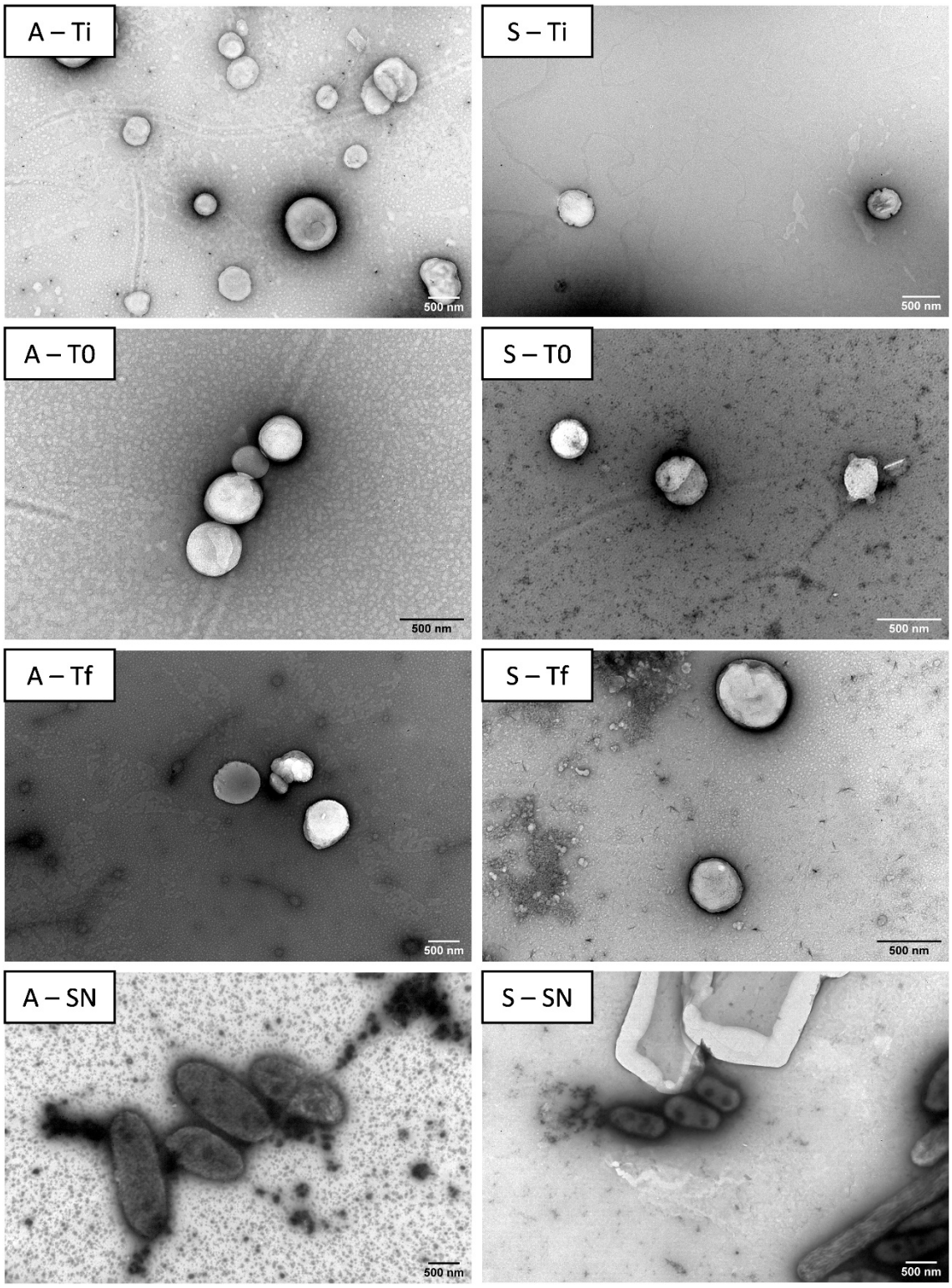

Figure 1 – Transmission electron microscope (TEM) photographs of both arachidic acid (A) and stearic acid (S) solid lipid nanoparticles (SLN) with Tween® 60. Ti – SLN after production, T0 – SLN after contact with rumen inoculum, and Tf – SLN after 24 h of incubation in the rumen inoculum, SN - supernatant.

Considering that only the SLN composed of stearic or arachidic acids with Tween® 60 were able to resist ruminal digestion, but that they were found to have the lowest %Lys, attempts to improve the latter were performed. These attempts included the use of a W/O/W double emulsion method of production [4], which rendered NP with similar values of %Lys (Table 7). It was also attempted to add the surfactant in the aqueous phase along with the Lys and to place the formulations at 4 ˚C immediately after production, to determine if the Lys entrapment would be improved (Table 7).

Table 7 – Lys encapsulation values (%Lys) for SLN composed of stearic or arachidic acids with Tween® 60, using the standard sonication method, the W/O/W double emulsion and sonication method where the final dispersions were immediately placed at 4 ˚C after production and where the surfactant was placed in the aqueous phase.

Lipid used	**Production method**	**%Lys (%)**
Stearic acid	Standard sonication-based	39 ± 5
	W/O/W double emulsion	33
	Sonication with immediate placement at 4 ˚C	28
	Sonication with the surfactant in the aqueous phase	30
Arachidic acid	Standard sonication-based	38 ± 6
	W/O/W double emulsion	34
	Sonication with immediate placement at 4 ˚C	37
	Sonication with the surfactant in the aqueous phase	30

However, none of these attempts appeared to improve the %Lys of the NP, thus the initial sonication-based production method was selected for further study, as it was the simplest method.

2.2.4. Nanoparticle behavior at high temperatures

Studying the alterations that NP undergo when subjected to high temperatures can provide very useful data, both to predict how they would react at high temperatures, such as those found in some industrial processes of feeds, and to characterize their stability over time. A DSC assay was performed to study the effects of high temperatures in the NP and its results can be seen in Table 8. The determined melting point for stearic and arachidic acids were 72.1 ˚C and 79.9 ˚C, respectively. The determined value was very similar when compared with the true value of 72.5 ˚C of stearic acid but a bit higher when compared to the value of 75.5 ˚C for arachidic acid [38], as expected since the components used were highly pure. Moreover, Lys appears to have no effect in any of the determined properties of the bulk materials, as expected considering its high melting point of 224.5˚C. In contrast, the NP structure presented both a decreased melting point and onset values, as well as a high RI value.

Table 8 – Differential scanning calorimetry (DSC) results for solid lipid nanoparticles (SLN) composed of stearic (S-SLN) or arachidic (A-SLN) acids with or without lysine (Lys), as well as controls of stearic and arachidic acids and its physical mixture with Lys.

Sample	ΔH[1] (J/g)	Onset (°C)	Melting Point (°C)	RI[2] (%)
SLN S without Lys	20.7	51.9	68.1	95
SLN S with Lys	21.3	59.1	68.4	97
Stearic acid	217.0	62.2	72.1	100
Stearic acid with Lys	219.1	59.5	71.4	100
SLN A without Lys	19.6	64.5	74.0	91
SLN A with Lys	20.1	67.7	74.5	93
Arachidic acid	217.0	68.0	79.9	100
Arachidic acid with Lys	219.1	68.7	78.0	100

[1]Enthalpy (ΔH)

[2]Recristalization index (RI)

The results obtained in the DSC analysis, regarding the melting points, are in accordance to the literature and can be explained by the presence of surfactants in NP and due to their increased surface area to volume ratio [7]. The almost ten-fold decrease in the enthalpy (ΔH) values when comparing bulk materials to the same materials in NP can also be attributed to the latter [7]. The NP also presented a high RI that indicates a reduced chance of the particles undergoing polymorphism, in theory allowing reduced chances of Lys expulsion during aging [39]. It can also be seen that Lys, when considering materials in NP, tends to slightly increase all the measured properties, probably due to interactions and alterations in the lipid matrix. These results, particularly the onset and melting point values, allows to state that the proposed NP can safely be used in processes that reach temperatures of 60°C without the risk of any degradation or damage, caused by temperature, to the

NP. This was expected, since the chosen lipids were selected for their high melting point.

2.3. Box-Behnken design optimization

The SLN composed of stearic or arachidic acids with Tween® 60 as surfactant were the only proposed NP capable of resisting ruminal digestion, despite presenting the lowest %Lys values among all the tested NP and were therefore selected for further studies. Since the attempts to increase the %Lys of these particles through changes in the production method were not successful, an alternative to improve %Lys was performed by optimizing the production method instead of altering it. This was achieved by applying BBD in the optimization of the SLN production method [8, 9].

Initially, the amount of lipid, the amount of surfactant, the amount of Lys and the amplitude of sonication were considered as the potentially more relevant parameters in the NP production method. To select the most influential parameters and to reduce the required number of runs of the experiment, several NP, using either stearic or arachidic acids with Tween® 60 as surfactant, were produced where these parameters were all fixed except for the one in study, which varied. The control parameters were: 100 mg of Tween® 60, 500 mg of fatty acid, 25 mg of Lys and 80% of sonication amplitude. All these parameters were evaluated (Tables 9 and 10) regarding their influence in terms of size, PdI and %Lys, as previously described.

Table 9 – Production parameter preliminary optimization results regarding stearic acid-based solid lipid nanoparticle (SLN), showing size, polydispersity index (PdI) and %Lys values for each tested combination.

Variable	**Size (nm)**	**PdI**	**%Lys (%)**
Tween® 60 - 75 mg	469	0.26	55
Tween® 60 – 100 mg	448	0.213	62
Tween® 60 – 125 mg	420	0.268	58
Stearic acid – 400 mg	453	0.21	54
Stearic acid – 500 mg	426	0.27	57
Stearic acid – 600 mg	449	0.28	57
Lys – 15 mg	438	0.23	36
Lys – 25 mg	452	0.25	43
Lys – 35 mg	432	0.25	36
Amplitude – 70%	385	0.28	61
Amplitude – 80%	453	0.23	60
Amplitude – 90%	465	0.22	58

Unless stated otherwise, 100 mg of Tween® 60, 500 mg of Lipid, 25 mg of Lys and 80% of sonication amplitude were used.

Table 10 – Production parameter preliminary optimization results regarding arachidic acid-based solid lipid nanoparticle (SLN), showing size, polydispersity index (PdI) and %Lys values por each tested combination.

Variable	Size (nm)	PdI	%Lys (%)
Tween® 60 – 75 mg	427	0.24	54
Tween® 60 – 100 mg	335	0.18	62
Tween® 60 – 125 mg	356	0.19	53
Arachidic acid – 400 mg	403	0.21	51
Arachidic acid – 500 mg	370	0.22	56
Arachidic acid – 600 mg	429	0.19	55
Lys – 15 mg	161	0.17	45
Lys – 25 mg	405	0.21	53
Lys – 35 mg	386	0.22	50
Amplitude – 70%	439	0.20	62
Amplitude – 80%	432	0.18	61
Amplitude – 90%	378	0.22	58

Unless stated otherwise, 100 mg of Tween® 60, 500 mg of Lipid, 25 mg of Lys and 80% of sonication amplitude were used.

Of the considered parameters, the most influential were found to be the amount of lipid, the amount of surfactant and the amount of Lys (Tables 9 and 10) and these three were therefore chosen to be optimized using a BBD. The influence of these parameters on %Lys and %LC was assessed, and their desirability function optimized (Figure 2). Table 11 shows the predicted optimal values that were rendered by the BBD as well as the %Lys and %LC values of the NP produced using the obtained values. It can be observed that values obtained were similar to the values predicted by the BBD, and in the

case of the stearic acid SLN the %LC obtained was even higher than the predicted values.

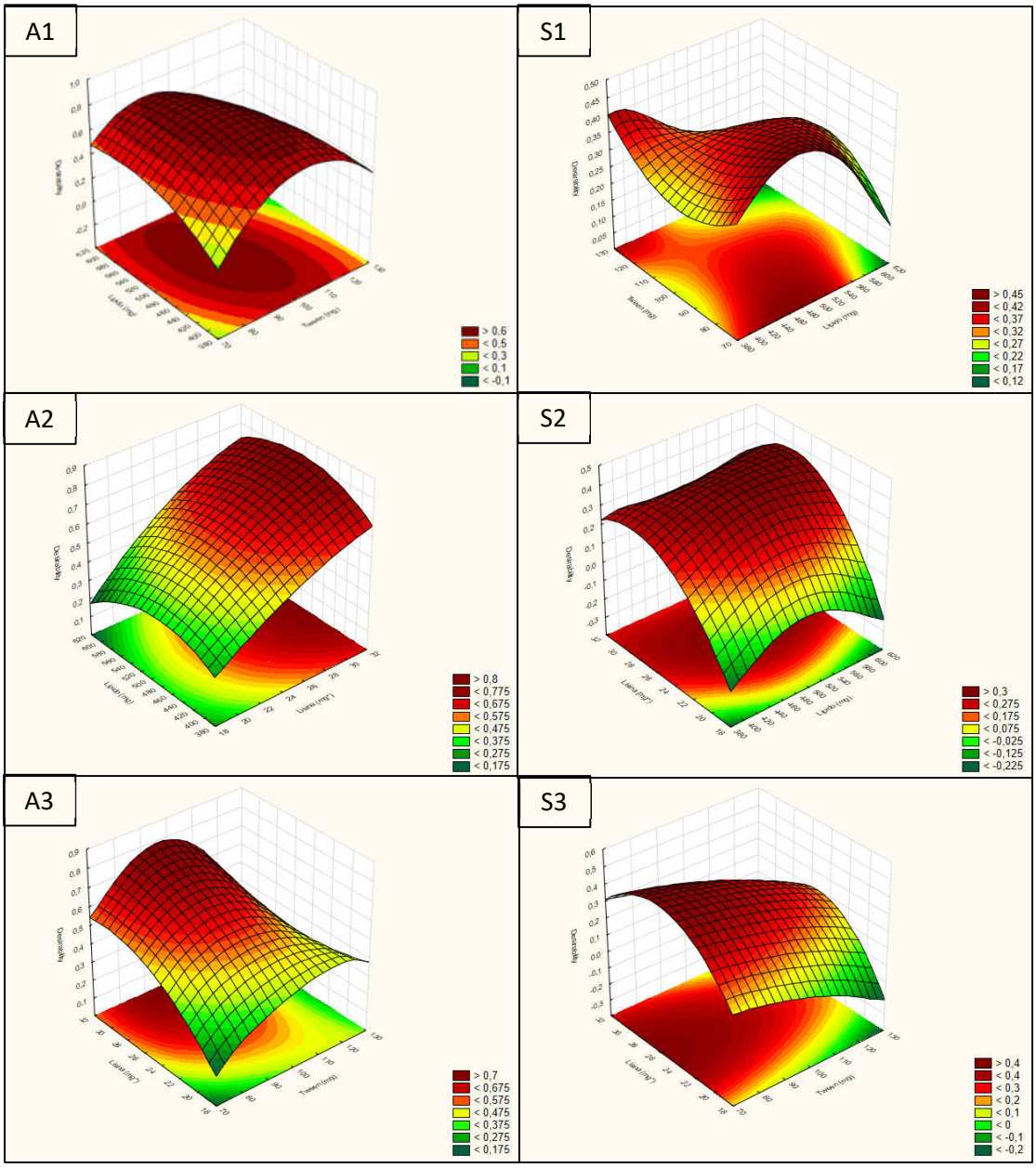

Figure 2 – Three-dimensional (3D) plots showing the influence of the independent variables on desirability, (A) arachidic acid solid lipid nanoparticle (SLN), (S) stearic acid SLN, (1) lipid amount vs surfactant amount, (2) lipid amount vs lysine (Lys) amount and (3) Lys amount vs surfactant amount.

Table 11 – Box-Behnken design (BBD) predicted results and results obtained using the predicted values of lysine (Lys) encapsulation efficiency (%Lys) and loading capacity (%LC) for both solid lipid nanoparticles (SLN) considered, (n=3).

SLN	Lipid (mg)	Surfactant (mg)	Lys (mg/mL)	%Lys	%LC
BBD predicted values for Arachidic Acid	503.72	95.87	6.82	66.1	3.3
BBD predicted values for Stearic Acid	400.01	96.01	6.82	58.7	3.5
Obtained values for Arachidic Acid				61.0 ± 2.1	3.1 ± 0.1
Obtained values for Stearic Acid				52.6 ± 0.9	5.0 ± 0.1

Applying a BBD, an optimization of the NP production parameters was performed, changing the lipid amount, the surfactant amount and the Lys amount added in the NP. The desirability function allows comparing and optimizing the effects of different, sometimes antagonist, parameters and was used in this work to maximize both %Lys and %LC. 3-D plots of combinations of the tested parameters vs desirability are shown in Figure 2. As expected, increasing the amount of Lys tended to increase the desirability function for both SLN tested, as this leads to more Lys being encapsulated and, therefore, increases both %Lys and %LC. It can also be observed that a lipid amount of around 500 mg and 400 mg for arachidic and stearic acid SLN, respectively, appears to have the highest desirability. It is most likely that these values achieve the highest possible %Lys without compromising the %LC, since the latter is inversely proportionate to the total mass of the NP. A similar explanation can be used for the amount of Tween® 60 that also appears to maximize the desirability function at the central value of 100 mg.

By interpolating the desirability function obtained with the BBD results, it is possible to calculate the optimal production parameters for which both %Lys

and %LC would be maximized and their respective predictive values. The optimal theoretical values for the tested parameters as well as the expected outcomes regarding %Lys and %LC are presented in Table 11. The results and their similarity to the real obtained values validate the BBD results [8], thus they can be classified as suitable models with no random tendencies. It can be seen that while arachidic acid SLN presents the highest %Lys, the %LC of stearic acid SLN is considerably higher, translating in less amount of the latter to be needed to supply with same amount of Lys than the former.

2.4. Cost-effective production of the nanoparticles

The results present in the previous sections indicate that the developed NP have the potential to be applied in ruminal-protection approaches, but further studies are required in order to one day bring this technology to the market. This section focuses on studying aspects of the production that could help the NP jump from the lab to the industry in the future.

It should be noted that the next assays were only performed using stearic acid, simply because this lipid is relatively cheaper and more available than arachidic acid, consequently rendering a more cost-effective productive of these NP.

A comparison of the effects of component purity and production method, standard organic solvent-free stirring and/or sonication and HPH, was performed (Table 12 and Table 13, respectively). All the NP were characterized in terms of size, PdI, zeta potential and %Lys, as described in **section 2.1.**.

It can be seen (Table 12) that none of the assessed characteristics were significantly affected when the lab-grade lipid and surfactant were replaced with less pure components, except for the %Lys that slightly increased, when considering the sonication production method.

Table 12 – Size (nm), polydispersity index (PdI), zeta potential (mV) and %Lys (%) values obtained for NPs produced using lab-grade and commercial lipids, n=3.

	Lab-grade	**Commercial**	**SEM**[1]	**P value**
Size (nm)	477	456	7	0.106
PdI	0.24	0.26	0.03	0.264
Zeta potential (mV)	-42	-35	4	0.111
%Lys (%)	61	64	1	0.041

[1]Standard error of the mean (SEM).

The lower purity of the NP components does not influence their properties, enabling a reduction in the overall cost of the formulation without compromising its quality. Additionally, the use of these components slightly increases the %Lys, from 61% to 64%, probably due to the fact that the less pure stearic acid used had a percentage of almost 40% of palmitic acid. The presence of these two solid lipids with different carbon lengths (C18 and C16, respectively) should create a less crystalline matrix with more imperfections where more Lys can be trapped [2].

A comparison between the standard lab-scale sonication-based production and a more scalable method based on HPH was also performed (Table 13) [40]. The results show that both production methods are able to produce SLN with similar properties regarding their size, PdI and zeta potential, with no significant differences being found between the NP. Comparing the %Lys of both methods, they are also statistically similar regardless of the number of cycles.

Table 13 – Size (nm), polydispersity index (PdI), zeta potential (mV) and %Lys (%) values obtained for nanoparticles (NP) produced by the sonication method and by the high-pressure homogenization (HPH) method with 1 (HPH-1), 2 (HPH-2) and 3 (HPH-3) sequential cycles, n=3.

Method	Sonication	HPH-1	HPH-2	HPH-3	SEM[1]	P value
Size (nm)	456	364	415	430	18	0.087
PdI	0.26	0.30	0.25	0.24	0.01	0.364
Zeta (mV)	-36	-37	-37	-39	2	0.823
%Lys (%)	64	59	55	54	2	0.052

[1]Standard error of the mean (SEM).

A tendency can be observed regarding the size of the NP, where a reduced number of cycles seems to reduce it, whereas increasing this number seems to produce NP with sizes more similar to the sonication-based production method. Despite no significant differences in the %Lys, a tendency can be observed as this value appears to decrease with increasing number of cycles. This decrease is probably due to the fact that when performing more than 1 cycle, the intermediate emulsions are placed at temperatures higher than the melting point between cycles [11].The latter could cause the NP to partly destabilize and release a portion of the encapsulated Lys, that is highly hydrophilic. Nevertheless, it can be seen that no significant differences exist, in any of the tested properties, when comparing the different production methods, indicating that it was possible to replicate the initial NP produced with a sonication-based method using an HPH-based method. The latter is much easier to scale up and thus enables a production on a much larger scale without compromising the NP properties. Considering that the objective is to achieve an industrial production, the single cycle HPH method was selected for future applications, since fewer cycles translate into less time and steps required to produce the NP. Additionally, a single cycle seemed to favor the Lys loading within the NP.

To ensure that the ruminal resistance of NP was not altered by a less pure composition and a different production method both standard sonication- and

HPH-produced NP were subjected to a ruminal resistance assay, as previously described. Furthermore, and to assure that NP resistance was achieved regardless of the animal's current diet, this assay was performed using rumen inoculum collected from cows fed diets differing in the base forage (maize silage or haylage) and forage:concentrate rations. No significant differences were found between NP after production (Ti), NP with contact with the ruminal media (T0) and NP after 24 h of incubation in the ruminal media (Tf) for both production methods and among all three diets (Figure 3).

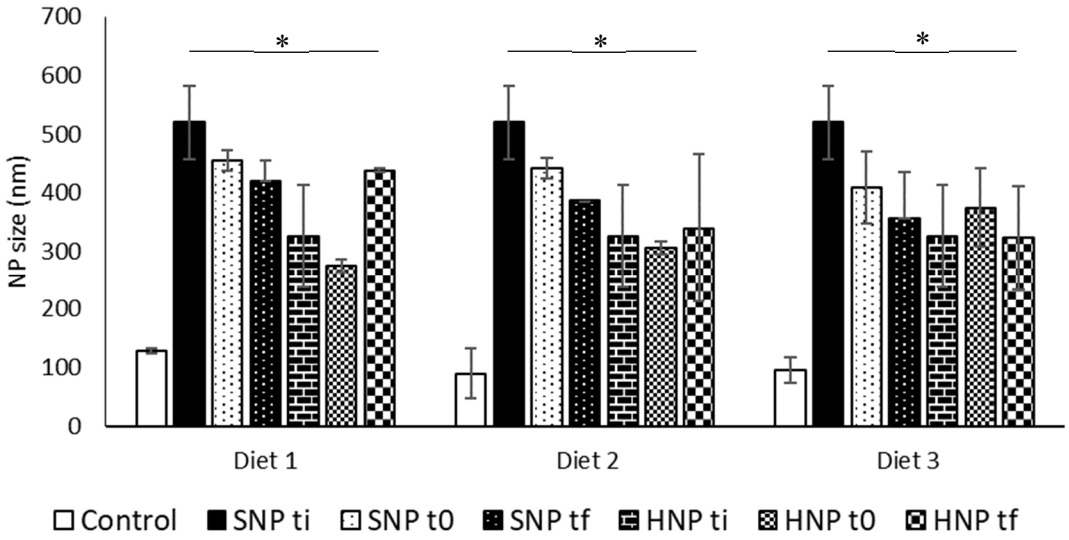

*Figure 3 – Nanoparticle (NP) particle size of solid lipid nanoparticle (SLN) produced by the standard method (SNP) and by high-pressure homogenization (HNP), where values are shown for the initial NP (Ti), for 0 h (T0) and 24 h (Tf) of incubation in the rumen inoculum. The values are shown for NP incubated in the rumen of cows fed with three different diets. Controls without NP were used. * denotes statistically significant differences from the controls (P ≤ 0.05). No significant differences were found between NP in either timepoint and the respective initial NP (P ≤ 0.05).*

The different production parameters considered, lower purity of components and NP production method, were not expected to influence the ruminal digestion of the NP as the lipid matrix, in theory, would not be greatly altered. However, it was still required to ensure that the introduced alterations did not interfere with the ruminal digestion of the NP, since this property is essential. These results (Figure 3) support the previous conclusions that SLN are indeed able to resist ruminal digestion, and prove that the newly proposed, less pure, HPH-produced NP are also capable of resisting ruminal digestion. Moreover, all

NP at all timepoints were found to be significantly different from the controls (rumen inoculum without any NP) indicating that the separation method was effective and that the results were not tainted by particles naturally present in the rumen.

2.4.1. Shelf-life stability

When considering a product, its shelf-life or the amount of time it can be stored while retaining its properties is of extreme importance to assess its applicability. An assessment of the stability in storage, or “shelf-life”, was also performed to determine for how long the NP remained suitable for use (Figure 4). No significant differences were found in either standard sonication produced or HPH produced NP for up to 29 days of storage, indicating that the tested NP could maintain their properties and remained viable for use within the tested time period.

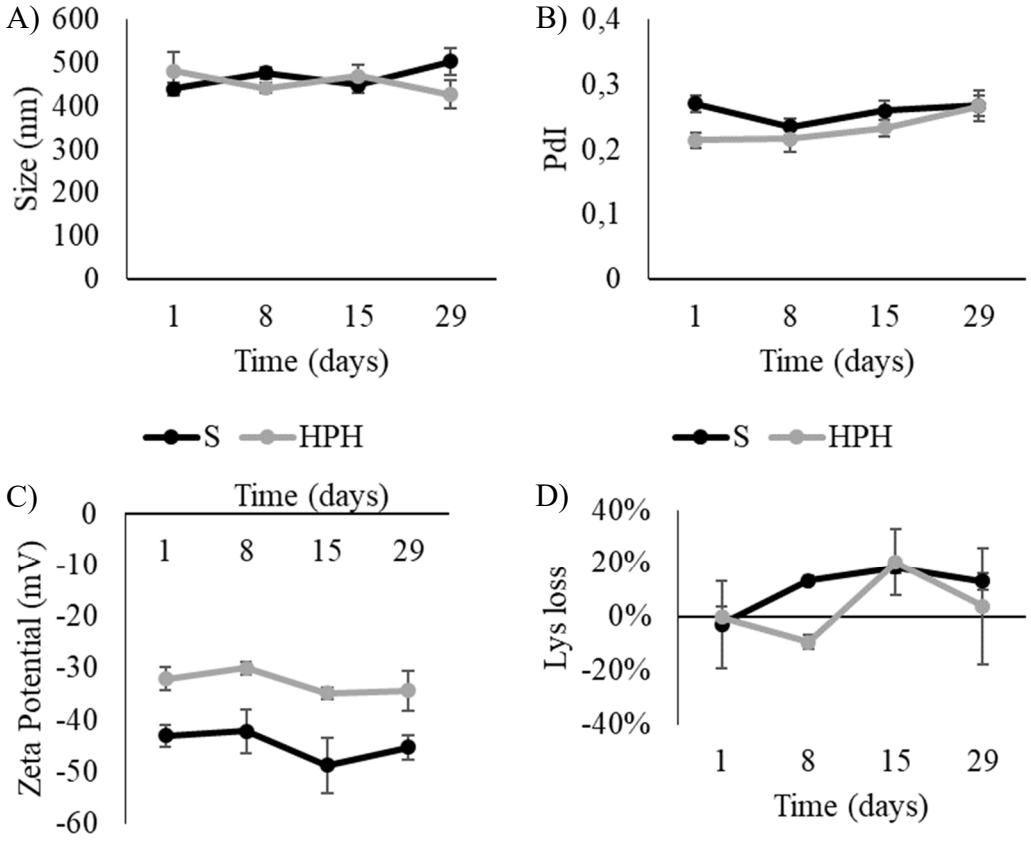

Figure 4 – Size (A), podlydispersity index (PdI) (B), zeta potential (C) and Lys losses (D) values for both nanoparticles (NP) produced by sonication method (S) and high-pressure homogenization (HPH) method over the period of 29 days of storage. No significant differences were found between day 1 of storage and any of the other tested timepoints ($P \leq 0.05$).

2.4.2. Nanoparticle lyophilization

The final NP formulations are aqueous dispersions, which might not be the ideal form for a commercial product, both for preservation and transport reasons. In an attempt to provide an alternative physical state for the NP, an assay was performed to assess the viability of their possible lyophilization, rendering a dry and much lighter final product. The results obtained in this assay (Table 14) indicate that the lyophilization process does not significantly alter the tested properties of the NP, except for the PdI.

Table 14 – Comparison of sonication and high-pressure homogenization (HPH)-produced stearic acid solid lipid nanoparticles (SLN), before and after lyophilization (Lyo). Values shown for size, polydispersity index (PdI) and zeta potential, n=3.

Formulation	**Sonication SLN**	**Sonication SLN Lyo**	**SEM[1]**	**P value**
Size (nm)	562	452	28	0.098
PdI	0.23	0.29	0.01	0.011
Zeta potential (mV)	-40	-36	1	0.051
Formulation	**HPH SLN**	**HPH SLN Lyo**	**SEM**	**P value**
Size (nm)	352	394	16	0.166
PdI	0.30	0.27	0.01	0.049
Zeta potential (mV)	-34	-32	1	0.355

[1]Standard error of the mean (SEM).

The alterations in PdI after NP lyophilization, in both SLN, could be the result of mild aggregation or NP erosion that widen, in the case of the sonication-produced SLN, or shorten the size distribution profile, in the case of the HPH-produced SLN. However, since the NP size remains similar, the later most likely occurs on a very small scale and should not be very influential. The tendency observed in the size reduction of the sonication based SLN also supports this theory. Another tendency was found when comparing the zeta reduction (in modulus) of the sonication produced SLN, however the value is still high (in modulus) and should not influence the stability of the resuspended formulation. The obtained results indicated that the NP could potentially be converted into a lyophilized form, with the inherent benefits, without compromising their efficiency. These findings should, however, be verified by performing more studies and assessing all NP properties after the lyophilization process to determine if they retain all of their original characteristics.

Conclusions

The existing products for Lys ruminal protection are ineffective because the degree of protection is low, with Lys degradations of 50% being registered [41], and no guarantee is given that all the Lys that escapes ruminal digestion is absorbed into the bloodstream. In this work a novel approach in animal nutrition was taken to overcome these limitations, relying on nanotechnology approaches to improve both Lys ruminal protection and improve the amount of Lys that reaches the blood. In order to achieve this objective, the delivery system would have to be able to carry Lys through the rumen while being produced in a scalable and cost-effective manner. In this chapter, the production and optimization of the NP were presented and discussed, and the results allow to conclude that, utilizing saturated fatty acids it was possible to design a system capable of by-passing ruminal digestion. In fact, SLN composed of stearic or arachidic acids, prepared with Tween® 60 as surfactant, resisted ruminal fermentation. The NP were also able to be loaded with Lys and optimized to maximize the amount that could be transported while attempting to reduce production waste. The proposed NP were capable of being loaded with 31 mg and 50 mg of Lys per g of product, for arachidic and stearic acid SLN, respectively. These loadings are lower than the ones of existing commercial products but could prove to be more effective if the NP present improved ruminal protection or intestinal absorption. Additionally, these NP also appear to be able to resist temperatures up to 60 ˚C, similar to the ones used in the compound feed manufacturing, enabling them to be used as supplements in feed products.

Moreover, SLN composed of stearic acid were also produced with components used as pharmaceutical excipients, instead of with the ultra-pure lab-grade ones, and with a scalable production method, without compromising any of their properties. Producing NP with these cheaper components, would be translated in a reduced production cost while a scalable method would enable for them to be more easily produced at an industrial scale. At this moment, arachidic acid-composed SLN were not completely discarded, as a full biological characterization of the NP was not yet performed and they could

prove to be superior to the stearic acid-composed SLN and, therefore, more suitable for the overall objective.

To summarize, SLN composed of stearic or arachidic acid with Tween® 60 as surfactant were produced, with the capability of resisting digestion in the rumen and to be loaded with Lys, making them a promising alternative to current AA rumen-protection technologies. Furthermore, the production of stearic acid SLN was achieved using cheaper components and an easily scalable production method, enabling a large scale and cost-effective production that facilitates the jump from the lab bench to the market.

In fact, with the ruminal resistance proved, these NP could be applied and tailored to fit a wide array of different needs, both in the delivery of other bio-active compounds and for applications in other ruminant animals. These particular NP were optimized for Lys delivery in dairy cows and present high potential to overcome the current limitations in this field, but the results presented in this chapter pave the way for the application of nanotechnology in countless other animal science approaches.

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Chapter 3. Nanoparticle biostability throughout the digestive tract and beyond: *In vitro* and *ex vivo* studies

Context

In the previous chapter it was proven that solid lipid nanoparticles (SLN), composed of arachidic or stearic acids with Tween® 60, were capable of resisting digestion by the rumen microbiota and being loaded with lysine (Lys), thus possessing adequate properties to fulfill the objective of delivering Lys across the rumen.

After demonstrating that the SLN could function as a rumen-protection delivery system, further characterization was performed to determine if they possess properties that would enable them to effectively deliver Lys to dairy cows. Thus, in this chapter, the stability of the SLN in the digestive tract following the rumen and in the bloodstream were evaluated. The assessment of the capability of SLN to effectively protect Lys from rumen fermentation was also performed. The stability of the SLN in the abomasum and the small intestine was evaluated using biomimetic media, to determine if they could reach the latter without compromising the NP's physical properties. Once in the small intestine, the SLN itself could be absorbed into the bloodstream, potentially increasing Lys bioavailability by improving its uptake, mainly due to their reduced size [1]. The SLN must also be assessed to guarantee that they are not harmful to the animal, especially at the intestinal level where they are expected to have more contact with the latter's cells. Once in the bloodstream, the SLN must be able to release their Lys content, thus being important to evaluate SLN stability in the blood. These studies were performed to discover if the optimized SLN possessed potential to overcome the current limitations of the existing rumen-protection technologies: poor ruminal protection and not guaranteeing that all Lys that escapes the rumen is absorbed.

This chapter focuses on ensuring that SLN Lys content is indeed protected in the rumen and on studying the stability of SLN in the remaining digestive tract, to determine if they reach the intestine unchanged, as well as in the blood, to determine if they can be destroyed thus releasing their cargo. A SLN biosafety study was also included to guarantee that the SLN are not hazardous to the animal.

3.1. Biomimetic and biological assay methods

3.1.1. Materials

Stearic acid and SPAN® 80 were purchased from Merck (© Merck KGaA, Darmstadt, Germany). Arachidic acid, Tween® 60, Tween® 80, poly(vinyl alcohol), L-lysine monohydrochloride, lithium carbonate, dansyl chloride, ethylenediaminetetraacetic (EDTA) acid disodium salt dihydrate, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton™ X-100, methylamine hydrochloride, triethylamine, sodium acetate, pepsin and pancreatin (from porcine pancreas) were purchased from Sigma-Aldrich (St. Luis, MO, USA). L-phenylalanine ethyl-ester hydrochloride was acquired from Fluka (Fluka Chemie GmbH, Buchs, Switzerland), acetic acid from VWR Chemicals (VWR International S.A.S., Fontenay-sous-Bois, France) and acetonitrile and methanol from Honeywell (Honeywell Riedel-de Häen AG, Seelze, Germany). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin (10000 U/mL) mix, fetal bovine serum (FBS) and amphotericin B (Fungizone™) were purchased from Gibco® (Invitrogen Corporation, UK). Fasted state simulated gastric fluid (FaSSGF) and fed state simulated intestinal fluid (FeSSIF) were purchased from Biorelevant.com Ltd (London, UK). Aqueous solutions were prepared with double-deionized water (Arium Pro, Sartorius AG, Göttingen, Germany).

3.1.2. Nanoparticle production

Both stearic and arachidic acid SLN with Tween® 60 as surfactant, were produced using the optimized sonication-based production method described in **Chapter 2**.

3.2.3. Lysine protection within the nanoparticles

SLN were incubated with rumen inoculum as described in **Chapter 2** but were not separated from the rumen's microbiota. After incubation, samples were lyophilized (LyoQuest -85 plus v.407, Telstar ® Life Science Solutions, Terrassa, Spain) for 72 h at -80 ˚C under a pressure of 0.4 mbar.

The lyophilized samples were transferred to borosilicate flasks and analyzed by near-infrared (NIR) spectroscopy. The NIR spectra were collected on a Fourier transform near infrared spectrometer (FTLA 2000, ABB, Québec, Canada) with an indium-gallium-arsenide (InGaAs) detector and controlled via Bomen-Grams software (version 7, ABB, Québec, Canada). Each spectrum was obtained from an average of 64 scans within the wavenumber interval of 10000 – 4000 cm^{-1} with a resolution of 8 cm^{-1} in diffuse reflectance mode. For each sample, a total of three spectra were collected and then averaged for further data analysis. The background was performed using a reference substance (Teflon).

A total of four sets of samples were considered: SLN produced with encapsulated Lys and SLN produced without encapsulated Lys but in which Lys was added after SLN formation, both incubated during 0 h and 24 h with the rumen inoculum. For both SLN, different amounts of Lys were added in order to achieve the same seven final concentrations of 0.0, 1.1, 2.2, 3.3, 4.4, 5.5 and 6.6 mg/mL of Lys. Duplicates of each concentration, for each set, were processed and analyzed to construct a validation curve per set.

The amount of Lys was predicted by modelling the acquired NIR spectra against the experimental values of Lys through several partial least squares (PLS) [2]. Before the application of any chemometric tool, the spectra were mean-centered. Different pre-processing techniques, including standard

normal variate (SNV) and Savitzky-Golay filter (with different filter widths, polynomial orders and first and second derivative) were tested individually and in combination to optimize the PLS models. The optimal number of latent variables (LV) was selected using the leave-one-out cross-validation procedure to prevent model over-fitting. All the PLS models were developed considering the whole spectrum mean centered. The developed PLS models were evaluated according to the root mean square error of cross-validation (RMSECV) and the cross-validation (R^2_{CV}). The RMSECV was calculated according to the following equation:

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{N} (\hat{Y}_i - Y_i)}{N}} \quad (1)$$

where N is the number of samples, Y_i is the experimental result for samples i and $\hat{Y}_i$ is the value obtained with the cross-validation for each sample.

All calculations were performed using Matlab version 8.6 (MathWorks, Natick, USA) and PLS Toolbox version 8.2.1 (Eigenvector Research Inc., Wenatchee, USA).

3.1.4. Nanoparticle stability in the abomasum

The abomasal medium was mimicked using fasted state simulated gastric fluid (FaSSGF) [3], prepared according to manufacturer specifications, with a pH of 1.6, supplemented with pepsin at 1 mg/mL. Both stearic and arachidic acid SLN were incubated, in triplicate, in the previously mentioned medium, at 39 ˚C, under light stirring [4]. The same medium without any SLN was also incubated and used as control, also in triplicate. Aliquots were collected from each replica at 0 h (T0) and at 1 h (Tf) of incubation [5, 6] for further analysis. Both samples and controls were assessed in terms of size using dynamic light scattering (DLS) and compared with the values obtained for SLN without any contact with the abomasal mimicking media (Ti).

3.1.5. Nanoparticle stability in the small intestine

The intestinal medium was mimicked using FeSSIF [3], prepared according to manufacturer specifications, with a pH of 5, supplemented with pancreatin at 3 mg/mL. Both stearic and arachidic acid SLN were incubated, in triplicate, in the previously mentioned medium, at 39 ˚C, under light stirring [4]. The same medium without any SLN was also incubated and used as control, also in triplicate. Aliquots were collected from each replica at 0 h (T0) and at 2 h (Tf) of incubation [6], for further analysis. Both samples and controls were assessed in terms of size using DLS and compared with the values obtained for SLN without any contact with the intestinal mimicking media (Ti).

3.1.6. Nanoparticle stability in the blood

The SLN stability in the bloodstream was assessed using two different media: FBS and fresh bovine blood. Both stearic and arachidic acid SLN were incubated, in triplicate, in both the previously mentioned medium, at 39 ˚C, under light stirring, protected from the light and sealed to avoid oxidation (only in contact with the atmosphere during aliquot collection). The same medium without any SLN was also incubated and used as control, also in triplicate. Regarding FBS, aliquots were collected from each replica at 0 h (T0) and at 3 h (Tf) of incubation, for further analysis. Bovine blood was collected from the jugular vein of Holstein Friesian cattle during their slaughter at PEC Nordeste - *Indústria de Produtos Pecuários do Norte*. Blood was harvested and stored in 0.5 L Schott flasks, previously coated with EDTA. The coating was achieved by rinsing the flask with 5 mL of a 0.5 M solution of EDTA in a flux chamber and then allowing the water to evaporate, depositing EDTA in the inner flask. Aliquots from SLN incubated with bovine blood were collected at 0 h (T0), 3 h (T1), 6 h (T2), 9 h (T3), 12 h (T4) and 24 h (T5) of incubation. Both samples and controls were centrifuged at 2000 x*g* for 15 min to separate blood components, and the supernatants collected for further analysis. All samples and controls, for both media, were assessed in terms of size using DLS and compared with the values obtained for SLN without any contact with any of the media (Ti).

3.1.7. Nanoparticle *in vitro* biosafety assay

The L929 mouse fibroblastic cell line was selected for the *in vitro* biosafety characterization of the SLN, as they are recommended for biomaterial safety evaluation for biomedical devices and food applications [7].

Cells were cultivated in DMEM, supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin mixture and 1% (v/v) amphotericin B, at 37 ˚C in 5% CO_2 atmosphere. The medium was replaced every three days and when cells were confluent, they were detached from the culture flasks with a scrapper (Nunc™ Cell Scrappers, Thermofisher Scientific; Waltham, MA USA). After this physical detachment, cells were centrifuged at 300 x*g* for 5 min in a Heraeus™ Multifuge™ X1R centrifuge (Thermo Fisher Scientific; Waltham, USA) and then resuspended in fresh DMEM. Counting of viable cells was performed using a Neubauer chamber (Improved Neubauer Bright-Line, Boeco; Germany) in a Motic® AE2000 Binocular Inverted Microscope (Motic Electric Group Co., Ltd; Xiamen, Fujian, China).

To assess the impacts of SLN in cellular viability a MTT assay was performed [8]. Cells were firstly collected and seeded in 96-well plates (5×10^4 cells per well) with 100 µL of fresh culture medium. After cellular adhesion, the medium was discarded and replaced by fresh DMEM medium, supplemented with SLN or free Lys. The SLN were added to achieve the final concentrations of 0.125, 0.25, 0.5, 1 and 2 mg of SLN per mL of medium, and free Lys was added to achieve the same concentrations that would be found in the respective mass of SLN. Cells with no SLN nor free Lys were included to be used as positive controls and cells incubated with 1% triton X-100 were used as negative controls. The cells were incubated, in quadruplicate, for all the aforementioned conditions for 24 h, at 37 ˚C in 5% CO_2. After incubation, the medium was completely discarded and replaced by 100 µL of a 0.5 mg/mL MTT solution. The plate was incubated for 2 h, at 37 ˚C in 5% CO_2, after which the MTT solution was discarded and 200 µL of DMSO was added to solubilize the formazan crystals formed inside the cells. Finally, the absorbance was read at 570 and 630 nm using a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instruments, Winooski, VT, USA). The absorbance at 630 nm was subtracted

from the absorbance at 570 nm to remove interferences from the media, and the cellular viability was calculated by comparing the treated cells with the control cells. The results were normalized and compared with the positive controls (in theory, 100% of viability).

3.1.8. Statistical Analysis

All statistical analyses were performed with IBM SPSS Statistics (SPSS 25.0, Armonk, NY, USA). Univariate one-way analysis of variance (ANOVA) was performed to compare multiple groups of independent samples. When the effect was statistically different ($P \leq 0.05$), the differences between the respective groups were compared with a post-hoc test (Pairwise, $P \leq 0.05$).

3.2. Ruminal protection of lysine encapsulated in nanoparticles

The ruminal microbiota is capable of striping Lys from within SLN without necessarily destroying them, and for this reason the previous obtained results (**Chapter 2**) are not sufficient to ensure that the SLN are capable of protecting the encapsulated Lys. Since a direct quantification of the encapsulated Lys was not possible, as stated in **Chapter 2**, an alternative method to assess the Lys protection within the SLN was required. A method based on NIR spectroscopy and relying in multivariate analysis, was considered and applied to evaluate the Lys protection of the proposed SLN.

The assessment of the SLN's protection of the encapsulated Lys during ruminal incubation was performed through the prediction of the Lys amount present in rumen inoculum using the respective NIR spectra. Several PLS models were developed, with the best PLS models being obtained through pre-processing the spectra with Savitzky-Golay filter (15 points filter width, second polynomial order and second derivative) followed by SNV and using 5 LV. The PLS models obtained for the prediction of Lys with and without encapsulation in the SLN before incubation with rumen inoculum (Figure 1.A and 1.C) revealed a coefficient of determination of R^2_{CV} higher than 0.75 and RMSECV lower than 6.6 mg of Lys, which indicates that it is possible to predict the amount of Lys

present in rumen inoculum. Regarding the PLS models obtained for the prediction of Lys with and without encapsulation in the SLN after 24 h of incubation with rumen inoculum (Figure 1.B and 1.D), different results were obtained. In the former case, the R^2_{CV} and RMSECV were 0.93 and 2.6 mg, respectively, while in the later the R^2_{CV} and RMSECV were 0.04 and 13.4 mg, respectively. These results indicate that the determination of the amount of Lys present in rumen inoculum through NIR spectra is possible and that Lys encapsulation in the SLN is efficient.

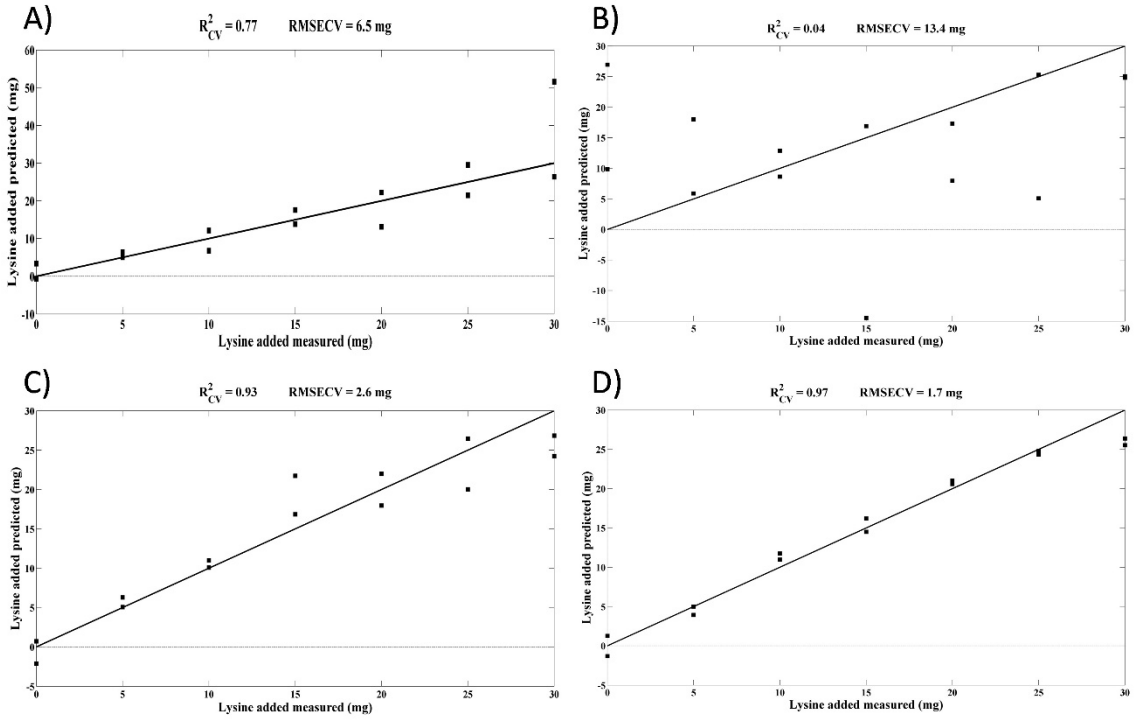

Figure 1 – Partial least squares (PLS) models obtained at T0 (0 h of incubation) for solid lipid nanoparticles (SLN) with lysine (Lys) added after production (A) or encapsulated during production (C) and Tf (24 h of incubation) also for SLN with Lys added after production (B) and encapsulated during production (D). Values of Cross-validation (R^2_{CV}) and Root mean square error of cross-validation (RMSECV) shown for each model.

The PLS models developed using the samples before incubation revealed that it is possible to determine the amount of Lys with and without encapsulation in the rumen inoculum, since high R^2_{CV} were obtained for both cases (Figure 1.A and 1.C). It was expected that the values of R^2_{CV} and RMSECV would be similar for the prediction of the amount of Lys encapsulated in the SLN or

without encapsulation. The results obtained were slightly different mainly because one of the samples with the highest amount of Lys (30 mg) without encapsulation predicted a higher amount of Lys. Nonetheless, the objective of these assays was not to develop an accurate and robust PLS models for the prediction of Lys in the rumen inoculum (to achieve this, more samples would be needed to validate the model) but to demonstrate that it is possible to determine the amount of Lys present in the rumen inoculum through NIR spectra. In this sense, the results obtained attest that it is indeed possible to quantify the Lys in the rumen inoculum.

The PLS models developed using the samples with and without Lys encapsulation and with an incubation time of 24 h were used to demonstrate the efficiency of Lys encapsulation in the SLN. The R^2_{CV} and RMSECV obtained for the PLS model using encapsulated Lys after 24 h of incubation, 0.93 and 2.6 mg respectively, were very similar to those obtained for the same samples of Lys encapsulated before incubation. For the samples without encapsulation after 24 h of incubation, the obtained R^2_{CV} and RMSECV, 0.04 and 13.4 mg respectively, revealed that the amount of Lys present was not able of being predicted. This could be associated to the chemical changes that occur in the rumen and in this sense may have contributed to the degradation/modification of Lys, most likely by the rumen's microbiota. Therefore, the results obtained with an incubation of 24 h indicate that the encapsulation of Lys in the SLN is efficient or otherwise the R^2_{CV} would be close to 0 and the RMSECV high.

3.3. Nanoparticle stability in the abomasum

In ruminant animals, after the reticulum-rumen, the digesta flows to the abomasum, which has an acidic pH. Incubation in abomasal conditions did not significantly affect neither arachidic nor stearic acid SLN, in terms of size, for up to 1 h of incubation (Table 1). A tendency was observed in SLN composed of stearic acid, with a slight increase in size, probably due to some adsorption of medium components. However, this increase is very small (less than 10% of the SLN size) and should, therefore, not be biologically relevant.

Table 1 – Average size (nm) of solid lipid nanoparticles (SLN) composed of stearic or arachidic acids, without contact with the abomasal medium, with contact with the abomasal medium but where no incubation was allowed to occur (0 h of incubation) and after 1 h of incubation in the abomasal medium, n = 3.

	SLN			**SEM**[1]	**P value**
		Stearic acid			
	No incubation	0 h	1 h		
Size (nm)	426	438	453	7	0.075
		Arachidic acid			
	No incubation	0 h	1 h		
Size (nm)	400	402	416	19	0.802

[1]Standard error of the mean (SEM).

The obtained results show that both stearic and arachidic acid SLN should be capable of resisting digestion in the abomasum, at least for 1 h. This stability is adequate to ensure that the SLN are capable of crossing the abomasum, considering that there is an almost continuous flow of digesta in this organ. Indeed, the high amounts of feed intake of high producing ruminants [9] render short abomasal clearances [10, 11], with half-times as low as 13 min in cattle [12].

The conclusion that the proposed SLN are capable of resisting digestion in the abomasum is also strengthened by the minimal digestion of lipids there [13], and by the low susceptibility of the selected type of lipid SLN to pH variations [14-16].

3.4. Nanoparticle stability in the small intestine

To ensure that all Lys that escapes the rumen is absorbed into the bloodstream, the ideal outcome would be that the SLN themselves be absorbed and afterwards release their contents to be used by the animal. Particles with

approximately 500 nm in size, similar to the proposed SLN, have been shown to exhibit an increased intestinal uptake in humans [1]. Nonetheless, similar studies in bovines have not been reported. For this reason, it is important to ensure that SLN are not altered by the digestion processes that occur in the small intestine and maintain their physical integrity and properties.

The incubation of SLN in intestinal-mimicking conditions did not significantly affect neither stearic nor arachidic acid SLN's dimensions (Table 2) after 2 h of incubation.

Table 2 – Size (nm) values of solid lipid nanoparticles (SLN) composed of stearic or arachidic acids, without contact with the intestinal medium, with contact with the intestinal medium but where no incubation was allowed to occur (0 h of incubation) and after 2 h of incubation in the intestinal medium n = 3.

	SLN			**SEM**[1]	**P value**
		Stearic acid			
	No incubation	0 h	2 h		
Size (nm)	485	456	448	12	0.139
		Arachidic acid			
	No incubation	0 h	2 h		
Size (nm)	374	350	401	18	0.211

[1]Standard error of the mean (SEM).

In the small intestine, digestion takes place at near neutral pH and with the assistance of several enzymes. For this reason, and to create a more realistic model, the intestinal media was supplemented with a pancreatic enzyme complex, containing amylase, trypsin, lipase, ribonuclease and protease. However, the incubation in this complex media did not significantly affect any of the SLN, which maintained their integrity and dimensions after 2 h of incubation in this media. This time period is used to determine digestibility in cows [17] and is also the indicated time period in an already existing *in vitro* duodenal model [18]. Considering that the proposed SLN are composed of

saturated fatty acids, their resistance in the small intestine should not be compromised by the presence of lipases, since these enzymes mainly digest triglycerides. In fact, the natural resistance of these fatty acids to digestion, across the bovine digestive tract, was the reason for their selection as components for SLN production. The natural resistance of stearic and arachidic acid to lipase digestion, as well as these SLN's natural resistance to pH variations [14-16], explains why the incubation in intestinal conditions did not affect the integrity or dimensions of the SLN.

The presented results (Table 2) indicate that the proposed SLN retain their physical properties after incubation in the intestinal media, potentially enabling them to be absorbed into the bloodstream.

3.5. Nanoparticle biosafety studies

The MTT assay was used to determine the potential cytotoxicity of the developed SLN (Figure 2). Free Lys showed no toxicity in all tested concentrations, while the SLN presented a significant cytotoxicity only at the highest concentration of 2 mg/mL. At lower concentrations, up to 1 mg/mL, the SLN presented high biocompatibility, reflecting approximately 80% and 100% of cellular viability, for stearic and arachidic acids, respectively.

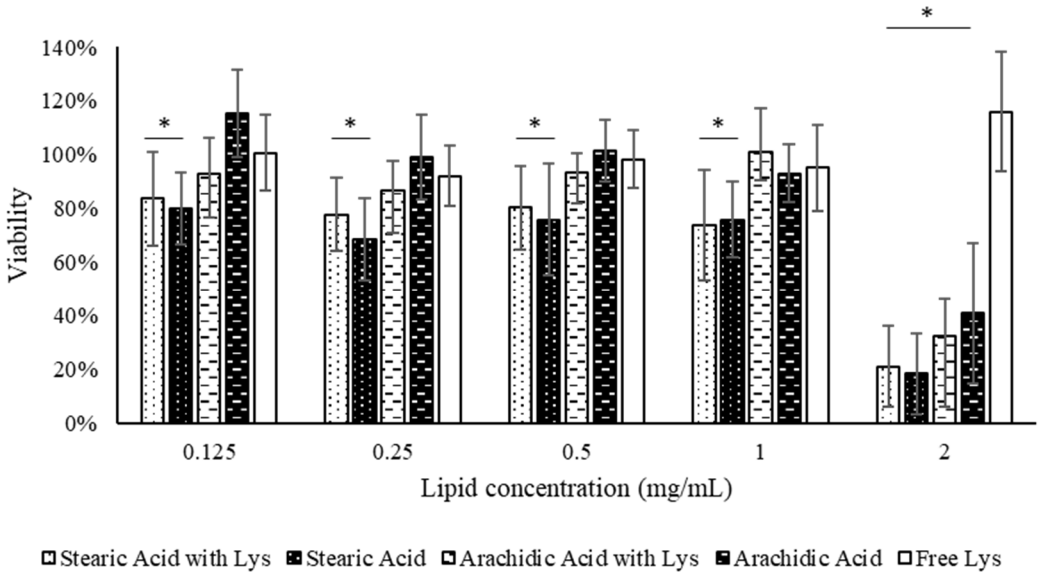

*Figure 2 – Cellular viability assay with L929 cells exposed to solid lipid nanoparticles (SLN) made of stearic or arachidic acids, with and without lysine (Lys), as well as free Lys, at increasing concentrations (0.125, 0.25, 0.5, 1 and 2 mg/mL of lipid). Free Lys concentrations were the same that were present in the respective Lys-loaded SLN. Values shown as mean ± standard deviation, n=3. *Statistically different (P ≤ 0.05) from the positive controls.*

All components used to produce the SLN are considered safe and possess high biocompatibility [14, 19]. The results obtained not only confirmed the safety of the used SLN components, but also proved that their combination and structure in SLN form also did not present a high toxicity. The viability obtained for stearic acid SLN were found to be statistically lower than the control, but the difference between these values was not higher than 20%, rendering this difference not biologically relevant [7]. The differences registered between stearic and arachidic acid SLN are not in accordance with the literature [20] that report similar biosafety among these fatty acids. Perhaps the longer chain of arachidic acid (when compared to stearic acid) interacts differently with the Tween® 60 or directly with cells and causes less damage to the latter. Regardless, both SLN present a considerably low toxicity in fibroblasts, when used in concentrations up to 1 mg/mL, making them safe for use as biomaterials in feed according to the existing regulations, which states that an effect or condition is only considered toxic when it reduces viability by 30% or more [7]. These results indicate that the proposed SLN are not inherently toxic and could potentially be used in animals. The performed cellular studies are an important tool to provide preliminary data on biosafety, thus reducing the

need to expose animals to several studies and tests. Further studies will of course have to be performed to ensure that these SLN are safe for use in animal nutrition.

3.6. Nanoparticle stability in the bloodstream

The stability in the abomasum and in the small intestine is very important to ensure that the SLN reach the latter without losing integrity or their Lys content. However, in order for Lys to be used by the dairy cow it must be released in the blood, becoming available for use [21, 22]. To assess if the SLN were capable of releasing their Lys content once in the bloodstream, a stability assay was performed, using two distinct media: FBS and fresh bovine blood. A first test, using FBS, was performed to determine if the interaction with blood contents, mainly circulating proteins, was enough to destabilize the SLN. Fresh bovine blood was used as a more complex and realistic model, after the tests using FBS.

Incubating SLN, of both stearic and arachidic acid, for 3 h with FBS (Tf) did not alter their dimensions when compared with the respective SLN after synthesis (Ti) or after contact with the medium (T0) (Table 3).

Table 3 – Size (nm) values of solid lipid nanoparticles (SLN) composed of stearic or arachidic acids, without contact with fetal bovine serum (FBS), with contact with FBS but where no incubation was allowed to occur (0 h of incubation) and after 3 h of incubation in FBS, n = 3.

	SLN			**SEM**[1]	**P value**
		Stearic acid			
	No incubation	0 h	3 h		
Size (nm)	476	475	444	14	0.199
		Arachidic acid			
	No incubation	0 h	3 h		
Size (nm)	424	402	427	11	0.266

[1]Standard error of the mean (SEM).

Incubating the SLN composed of stearic acid in fresh blood appears to cause a slight reduction in size over time (Table 4). Significant differences were found between the initial SLN and all incubated SLN, whereas among the latter there were only differences after 24 h of incubation. Additionally, no differences were found between controls and these SLN after 24 h of incubation in the blood. Regarding arachidic acid SLN, significant differences are only found after incubating them for 9 h or more. Furthermore, 24 h of incubation still rendered differences from the controls.

Table 4 – Size (nm) values of solid lipid nanoparticles (SLN) composed of stearic or arachidic acids, without contact with blood, with contact with blood but where no incubation was allowed to occur (0 h of incubation) and after 3 h, 6 h, 9 h, 12 h and 24 h of incubation in blood, n = 3.

SLN		Size (nm)	SEM[1]	P value
Control		6[a,*]	3	
Stearic acid	Without contact with the blood	454[b]	38	<0.001
	After 0 h of incubation	361[b,c]		
	After 3 h of incubation	295[c]		
	After 6 h of incubation	293[c]		
	After 9 h of incubation	278[c]		
	After 12 h of incubation	275[c]		
	After 24 h of incubation	11[a]		
Arachidic acid	Without contact with the blood	401[b]	21	<0.001
	After 0 h of incubation	402[b]		
	After 3 h of incubation	325[c,e]		
	After 6 h of incubation	377[b,e,f]		
	After 9 h of incubation	294[c,d]		
	After 12 h of incubation	326[c,f]		
	After 24 h of incubation	243[d,#]		

[1]Standard error of the mean (SEM);

*This value is indicative of no present NP, as they were not added to the control;

#This value is not indicative of the total replicate populations, as in some of them no NP were detected, indicating a possible destruction;

Different superscript letters indicate significant differences ($P \leq 0.05$) between controls and each SLN at all timepoints. Stearic or arachidic acid SLN were compared only with the controls and not with each other.

The differences in the results obtained for the incubation in FBS and bovine blood might be explained by the fact that FBS is inactivated by heat during its production [23] and this inactivation could prevent enzymes from functioning properly or damage proteins important for SLN metabolization and degradation. Additionally, the diameter of the tested SLN makes them likely to

be phagocyted by the circulating macrophages [24, 25], being most likely destroyed within their lysosomes and, in theory, releasing their Lys content. The slow degradation of SLN, particularly of stearic acid composed SLN, in the blood strengthens the hypothesis that, once in the blood stream, they would release their Lys content where it can be used by the animal. A slow degradation of the SLN would imply a release of Lys over time, which could benefit milk production, due to the mammary gland's ability control specific AA influx [26, 27]. Since this gland has the ability to regulate blood flow and specific AA extraction, it could increase the transport of other AA when Lys, a limiting AA, is available thus improving production. A slow, over time, release of Lys into circulation would increase its availability for milk component synthesis. At the same time, Lys that remain within the SLN would remain protected from the natural occurring degradation and metabolism that AA suffer in the bloodstream or by some organs during circulation [21, 28]. On the other hand, a burst release could lead to a temporary excess of Lys in the blood that wouldn't be only used for milk production. This would cause an increase in Lys degradation or mobilization by other organs, reducing the overall Lys available to the mammary gland. The latter could lead to a reduction of the amount of Lys, supplied by the SLN, that would be used in milk component synthesis, thus reducing efficiency of the Lys delivery system. Further studies are required to validate this hypothesis, but the results presented indicate that SLN, particularly stearic acid SLN, were completely degraded in the blood, releasing their Lys for use by the animal.

Conclusions

The results in this chapter showed that the proposed SLN, composed of stearic or arachidic acids with Tween® 60 as surfactant, were capable of protecting their Lys content from the ruminal microbiota and keep their stability in media that simulates abomasum or small intestine conditions. Unlike other rumen-protected strategies, the NP were designed not to release their Lys content in the intestine, but instead for themselves to be absorbed into the bloodstream.

This alternative approach could also improve the overall efficacy of the SLN, by guaranteeing that all the Lys is delivered to the blood and increasing its bioavailability.

Additionally, the *in vitro* assays performed showed no inherent toxicity for both stearic or arachidic acid SLN, according to the existing regulations [7] and could therefore be considered for applications in living animals.

Both SLN of stearic and arachidic acids were found to be degraded in the blood over 24 h, particularly stearic acid SLN that were completely destroyed after this period of time. This enables the SLN, once in the bloodstream, to release their Lys content for milk component synthesis. Further studies should be performed to determine if this release occurs in a controlled manner overtime, which could also improve the nitrogen-usage in the animal by increasing the Lys-usage efficiency.

To summarize, both proposed SLN (composed of stearic or arachidic acids with Tween® 60 as surfactant) were capable of protecting their Lys content while crossing the rumen and resisting digestion across the remaining bovine digestive tract, without losing their integrity. Once in the bloodstream, the SLN were capable of releasing their Lys content in a controlled manner, therefore suppling it to the animal. Furthermore, the proposed SLN did not present an inherent toxicity, enabling them to be safely considered for applications in animal science.

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Concluding remarks

The objective of this work was to develop a novel rumen-protection amino acid (AA) delivery system, based on nanoparticles (NP), to increase the supply of lysine (Lys) to high production dairy cows. To achieve this, the proposed NP would have to be capable of protecting Lys across the bovine digestive tract and delivering it to the bloodstream, to then be used for milk production. Throughout this work several type of lipid NP were produced and characterized in multiple aspects, all of which essential to ensure that they could fulfill their objective as an AA-delivery system for dairy cows. The main conclusions that can be taken from this work are:

- A Lys quantification method, based on high-performance liquid chromatography with fluorescent detection, was developed and validated to accurately determine the amount of Lys that was loaded into the NP;
- Solid lipid nanoparticles (SLN), produced with an organic solvent-free method, composed of stearic or arachidic acid using Tween® 60 as surfactant, were able to resist ruminal digestion and protect their Lys content from the microbiota;
- Both SLN composed of stearic or arachidic acid were optimized, using a Box-Behnken design, to maximize their Lys content, reaching encapsulation efficiencies of 53% and 61%, respectively;
- These two SLN were also capable of resisting temperatures of at least 60 ˚C without risk of suffering any reorganization or destruction of their lipid matrix, enabling them to undergo the temperatures achieved in some industrial feed processing;
    - Additionally, SLN composed of stearic acid were capable of being produced using cheaper components and with an easily scalable production method, based on high-pressure homogenization, without compromising their properties;

    - These stearic acid SLN were also stable for at least a month of storage, in which their physical properties and Lys content were not altered;
- Both stearic and arachidic acid SLN were capable of resisting digestion in abomasum and small intestine mimicking media, maintaining their integrity and physical properties;
- The selected SLN were also not inherently toxic, in an *in vitro* viability study using fibroblasts, enabling them to be considered for *in vivo* applications;
- Once in the bloodstream, both SLN were degraded within 24 h, thus releasing their Lys cargo to be used by the animal for milk component synthesis;

Further studies are still required to validate the *in vivo* efficacy of the proposed SLN in supplying Lys to the animal, but the results presented throughout this work indicate that they are a promising alternative technology to deliver Lys in a rumen-protected manner. Furthermore, this technology, once properly validated, could easily be applied in the delivery of other AA or biomolecules to ruminant animals, paving the way for countless other approaches, based in nanotechnology, in the field of ruminant feeding and nutrition.

Future work and perspectives

Context

The results presented in this work indicate that the developed solid lipid nanoparticles (SLN), composed of stearic or arachidic acid with Tween® 60 as surfactant, possess the required properties to function as a rumen-protected lysine (Lys) delivery system. Additionally, these SLN were able to maintain their integrity in media designed to mimic the abomasum and the small intestine, being slowly degraded in the bloodstream, releasing, in theory, their Lys content.

However, in order to properly assess and validate the efficiency of the proposed system in improving Lys bioavailability, more studies are still required. An evaluation of the SLN absorption at the intestinal level is essential to ensure that they are capable of reaching the blood, where the release of Lys will occur. The latter assays can most likely be performed *in vitro* but, regardless of how promising the results might be, subsequent studies will have to be performed *in vivo*. Efforts were made during this project to reduce the number of assays to be performed in animals, but no *in vitro* assay is capable of accurately mimicking them or provide the same information. For the purposes of this work, *in vivo* assays are essential both to evaluate the efficacy of the Lys delivery SLN but also to determine if their ingestion in any way impairs normal animal health and wellbeing.

On a different scope, considerations for the industrial production and application of these SLN should also be taken into account. The results presented in **Chapter 2** showed that the stearic acid SLN could be produced with cheaper components and a more scalable production method but going from the lab bench to the market requires more.

This section focuses on further studies that are required to validate the proposed SLN as potential ruminal protection alternatives, as well as on studies that could be relevant for the industrial production of these SLN.

Intestinal absorption of the nanoparticles

Evaluating the intestinal uptake of SLN is very important when considering oral delivery applications and could be the difference between a success or failure of the delivery system. Considering that the objective of the SLN would be that they would be absorbed in the intestine to ensure that all the Lys that escapes the rumen reaches the bloodstream, their uptake must be assessed.

To address this issue, the Ussing chamber system was considered. In fact, during this PhD a three-month period was spend in the University of Hohenheim learning this technique so that it could be applied in this work.

The Ussing method utilizes *ex vivo* tissues and enables the study of the interaction and uptake of molecules or particles through those tissues. The system was initially developed to study ion transport through epithelial tissue but has since been applied to study transport of other molecules or drugs [1, 2]. It consists of two chambers separated by a thin membrane of living epithelial tissue, which is composed of naturally polar cells and has a mucosal and a serosal side. Both chambers are filled with the same volume of a rinsing solution to remove any driving force that would influence transport across the tissue. The transport of a specific molecule can be measured by placing it in one chamber (usually the mucosal side) and quantifying the amount that crosses to the other side over a defined period. Four electrodes are also connected to the tissue: two voltage electrodes that register any electrical variations that occur during transport and two current electrodes that inject current into the system and allow the latter to accurately measure the changes in voltage caused by ion transport [2, 3]. This, coupled with transport evaluation, allows to determine if the transport occurred with electrical changes to the cellular membranes or not [4].

The Ussing chamber system could be applied to evaluate the amount of SLN that are absorbed at intestinal level, using extracted living tissue of bovine intestine as the membrane between the chambers. A quantification of Lys that crosses would enable to determine the efficiency of the SLN in delivering their

cargo to the bloodstream. Furthermore, this experiment could be coupled with a dynamic light scattering (DLS) assay to detect the presence of SLN on the serosal side of the membrane. Should they be present in this side, it would mean that the SLN were absorbed without losing their integrity. On the contrary, if they were not present but Lys was still transported, it would mean that the SLN would be destroyed in the process of delivering Lys to the blood.

An alternative assay to evaluate SLN uptake could be the use of existing intestinal cellular models [5, 6]. A similar model using bovine intestinal cells could also be constructed [7], although this would be a much more complex process. These models are not as realistic as using the actual tissue, since the cellular organization and 3D architecture are not the same, but they are also capable of providing valuable information regarding intestinal absorption.

In vivo validation of the nanoparticles

Even with a good intestinal absorption, registered using *ex vivo* studies, an *in vivo* efficacy assay would always be required to properly validate the proposed SLN. No matter how complex or advanced the *in vitro* and *ex vivo* models are, the existing technologies are not capable of fully mimicking or accurately replicating all the mechanisms of a living organism. An *in vivo* trial would thus be important to assess the effects of dietary supplementation with the formulated SLN in milk production and composition and to verify their safety for the animal.

In such study, and in order to estimate the rate of the Lys release in the blood and the effects on milk protein synthesis, it would be useful to determine the arteriovenous differences (AVD), which are commonly used to determine the absorption by the mammary gland [8, 9]. Additionally, feed intake should be measured and rumen fermentation parameters determined to evaluate effects of SLN on rumen function.

Industrial considerations of nanoparticle production

Some aspects regarding the industrial production of the SLN were already considered and evaluated in **Chapter 2**, but before this technology can become a rumen-protected Lys product, other considerations are still required.

It was shown that is possible to produce SLN using cheaper components and by an easily scalable production method, thus reducing the overall costs and enabling production at a larger scale, without compromising their properties. However, producing SLN in large volumes requires specialized equipment with some specific requirements, such as working temperatures and pressures. To study the possibility of producing SLN in a large scale, a pilot test was performed using a high-pressure homogenization (HPH) system with a much higher volume capacity. This study was performed using a Rannie™ model Bluetop (Copenhagen, Denmark) homogenizer, at the *Escola Superior Agrária de Coimbra* of the *Instituto Politécnico de Coimbra*, to produce 5 L of SLN dispersion. The formulations produced using this homogenizer possessed properties similar to the ones produced in the laboratory, indicating that a similar model could be used in the production of SLN (Table 1), in terms of size and zeta potential. In fact, this larger scale production even slightly increased the amount of loaded Lys.

Table 1 – Size (nm), polydispersity index (PdI), zeta potential (mV) and %Lys (%) values obtained for SLN of stearic acid with Tween® 60 produced using the standard sonication method and the larger scale high-pressure homogenization (HPH) method, n=3.

	Standard	**HPH**	**SEM**	**P Value**
Size (nm)	456	950	138	0.054
PdI	0.26	0.34	0.01	0.005
Zeta potential (mV)	-36	-40	1	0.172
%Lys	64	70	1	0.022

However, this model of homogenizer was not capable of being maintained at a specific temperature. This did not appear to influence the production of the aforementioned volume, but when producing much larger volumes, it could become an issue, especially when considering the high melting point of stearic acid. An impossibility to heat the HPH system could cause a solidification of the components inside the system overtime and with repeated use, leading to a blockage. Ideally, the system used to produce SLN should be able to be kept at a temperature above the melting point of the components, to prevent malfunctions and a continued production.

Another aspect that was addressed was the capability of removing the water from the SLN formulation, without destroying them or compromising their integrity, which would lead to an unwanted expulsion or loss of their Lys content. Considering that the proposed formulations, in their final form, are approximately 90% water, some considerations must be made regarding their industrial production, particularly when considering the transport of the product. To attempt to address this issue, a study, present in **Chapter 2**, was performed to determine if the SLN could be lyophilized without compromising their properties. Lyophilization is a standard method for water removal of NP formulations in the laboratory [10, 11] but may not be the most suitable method to achieve this in an industrial setting. Lyophilization is a complex and usually time-consuming method that requires very specific conditions, particularly very low temperatures and pressures [12]. Such requirements make this method very expensive and sometimes unsustainable in the processing of industrial products [13]. Additionally, the standard lyophilization equipment is only capable of processing small amount of volumes, making it a non-viable method when considering the high amounts of feed that dairy cows ingest.

Spray drying is an alternative method for producing a dry powder from a liquid solution or suspension, which does not require vacuum but has the need for very high temperatures [14]. This method has inclusively been proposed for nanoparticles production [15, 16]. However, this method is also considerably expensive and proper economic studies should be performed before

considering it as an option for industrial scale drying of products. Other alternatives, such as vacuum foam drying [17] or microwave freeze drying [18] have also been considered, but their requirements for very low pressures and high temperatures, respectively, also put into question their economic viability.

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Appendix

Basic principles of the used methods

Nanoparticle formulation production

To achieve a safe and effective nanoformulation while simultaneously allowing for a cheap and scalable production method this work focused in lipid nanoparticles and three different production methods were assessed.

Organic solvent-free stirring and/or sonication

In this method, both the lipids and surfactants are melted and combined with an aqueous phase that forms an emulsion. This is then homogenized to form a microemulsion that is afterwards sonicated to render a nanoemulsion, which after cooling will crystalize and create a colloidal suspension of NPs. Studies have also been performed where the intermediate step, of homogenization to form a microemulsion, is skipped and only the sonication is performed [1]. Both the surfactant and the active molecule can be added in the lipid or the aqueous phase, depending on their hydrophobicity [2, 3]. This method is more suitable to encapsulate lipophilic molecules but can also be used with hydrophilic molecules [4].

In this work, three types of lipid nanoparticles were produced using this method: solid lipid nanoparticles (SLN) – NP composed of lipids that are solid at body temperature [1], nanostructured lipid carriers (NLC) – NP composed of a mixture of lipids that are solid and liquid at body temperature [5] and multiple lipid nanoparticles (MLN) – NP composed of a mixture of solid and liquid lipids that possess large water vacuoles inside [6].

W/O/W double emulsion

In this method, lipids are usually dissolved in an organic solvent and a small amount of an aqueous solution of the selected molecule and surfactant [7, 8]. This mixture is then sonicated to form an aqueous emulsion in oil. Afterwards,

a greater volume of an aqueous solution of surfactant is added and again sonicated to create the final water in oil in water emulsion [9]. The latter is then left under stirring to allow solvent evaporation and NP crystallization during cooling [10]. In theory, hydrophilic molecules remain in the interior aqueous vesicles, making this method more suitable to encapsulate this type of molecules [4, 7].

High-pressure homogenization (HPH)

This method can be performed both at high (hot HPH) or low (cold HPH) temperatures [11-14]. In the hot HPH method, a pre-emulsion of lipids and surfactants is formed by melting and stirring the components in water before homogenization. On the other hand, in the cold HPH method, the lipids and surfactants are melted together and rapidly ground under liquid nitrogen and stirred in a cold aqueous surfactant solution to create a pre-suspension. Afterwards, both the pre-emulsion or pre-suspension are placed in a high-pressure homogenizer (regulated to be at an adequate temperature for each method) to be processed, rendering either a nanoemulsion that will crystalize into a nanosuspension (hot HPH) or a nanosuspension (cold HPH). In both cases, the homogenizing conditions can differ, with different number of cycles and different pressure values being used [11]. This method is very easily scalable and the cold HPH method is also suitable for the encapsulation of temperature sensitive and/or hydrophilic molecules [4, 13].

In this work, only SLN were produce using this method [11].

Box-Behnken design

Multivariate techniques have been frequently applied in the optimization of several methods with a wide array of different applications, among which the production of NP can be found. These techniques are superior to univariate studies because they are capable of observing any synergism or antagonism that might exist between different parameters that would not be observed in the latter tests. Moreover, multivariate analysis also enables a reduction in the number of required experiments to produce the necessary data, decreasing

both the time and reagents required to draw conclusions. These methods comprise two kinds of variables: factors and responses, the latter being the outcome results that depend on different combinations of the former. By correlating both the responses and the factors, it is possible to mathematically determine the ideal value of each factor that would render the optimal responses. Box-Behnken designs (BBD) are among such techniques and can be classified as nearly rotatable second order designs based on three-level incomplete factorial designs and have been found to be more efficient than other response surface designs [15, 16]. Table 1 shows a standard block diagram for a Box-Behnken design with 3 factors and 3 levels, while Figure 1 shows its graphical representation. As it can be seen, BBD has the advantage of not containing any combination where all factors are at their maximum or minimum values, making suitable to avoid experiments under extreme conditions [17].

Approaches using BBD to optimize NP production are numerous [15, 18, 19] and were selected for this work for their combination of high efficiencies with significantly reduced experiments being required.

Table 1 – General block diagram for 3-factor Box-Behnken designs.

Run	X1	X2	X3
1	-1	-1	0
2	+1	-1	0
3	-1	+1	0
4	+1	+1	0
5	-1	0	-1
6	+1	0	-1
7	-1	0	+1
8	+1	0	+1
9	0	-1	-1
10	0	+1	-1
11	0	-1	+1
12	0	+1	+1
13	0	0	0
14	0	0	0
15	0	0	0

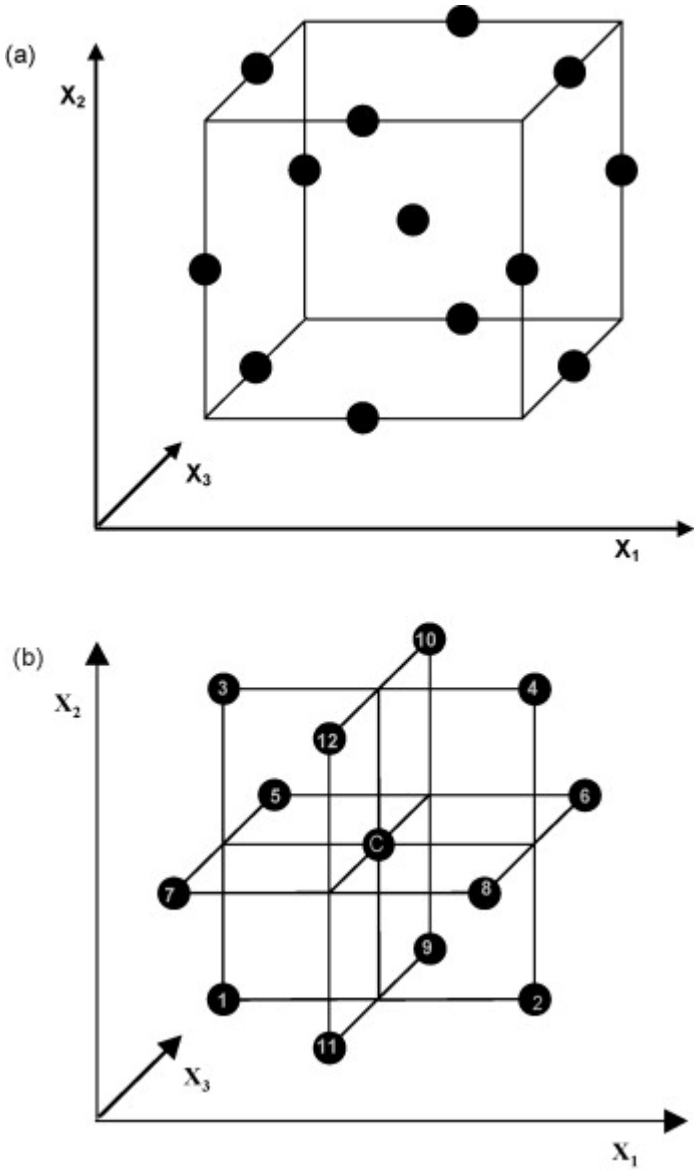

Figure 1 – Graphical representations of a BBD model for 3 factors and 3 levels, showing center points (C) and the midpoints of the edges (1-12) in b) [17].

Nanoparticle characterization

In order to compare the different NP formulations and to evaluate their potential for specific applications, both their physical properties and their biological properties need to be assessed.

Dynamic light scattering

Dynamic light scattering (DLS) is usually used to assess the mean hydrodynamic diameters and size distribution profiles of particle suspensions, relying on the fact that particles in suspension are in constant movement and that this follows the Brownian movement [20, 21]. In this technique, a laser is focused on the suspension and the light diffracted by this is registered over time. Considering that different size particles move at different speeds and that slower particles diffract more light (because they are slower to leave the laser beam's path) the apparatus is able to determine both the mean size and the size distribution profile of the suspension, taking into account the viscosity, temperature and refractive index of the medium [22, 23].

Both size and size distribution profile are very important properties of a NP formulation and considerably affect their intestinal absorption, circulation time and cellular uptake.

Electrophoretic light scattering

Electrophoretic light scattering (ELS) is based in DLS but instead allows for the measurement of the NP surface charge and their interaction with the surrounding medium [23]. Charged NP interact with the ions present in the media and create an unequal distribution of the latter, creating two layers of ions around the NP: the inner layer is formed by strongly bound ions (Stern layer), while the outer is formed by ions that are loosely bound to the inner layer (diffuse layer). The charge of this theoretical outer layer is very stable and is often defined as the Zeta potential of the NP. Zeta potential is usually measured by ELS, by placing two electrodes (perpendicularly to the DLS laser's path) and applying electrical potential to them. This causes the particles to move towards the electrodes according to their charge in DLS. Their charge is then determined based on their movement speed and direction [23, 24].

Zeta potential values are important to characterize a NP formulation, because they are associated with its stability: high values, in modulus, are usually

associated with higher repulsion between particles, rendering more stable suspensions that are less likely to aggregate.

Transmission electron microscopy

Transmission electron microscopy (TEM) consists of transmitting a thin beam of electrons through a sample. The electrons' interaction with samples is then registered to form an image with very high resolution [25]. This resolution is much higher than it is possible to obtain with an optical microscope because the de Broglie wavelength of electrons is considerably smaller than that of light. TEM images are black and white photographs, whose different contrasts usually depend on different thickness and/or density of the present components. Because of this, and to further increase image quality, contrast agents are sometimes used [24].

Regarding nanotechnology, TEM images are important tools to analyze size of NP but render even more information, particularly regarding their morphology and structure, that cannot be assessed by other commonly used methods such as DLS.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) consists in measuring the amount of energy that is required to increase the temperature of a substance or mixture of substances [26]. This is achieved by supplying energy to both a sample and a control until the temperature of both are elevated by a specified amount of degrees. The apparatus then registers the supplied energy to the sample over time and compares it to the control. Changes in the structure or arrangement of matter of the samples will lead to differences in the energy required to increase the temperature at these moments [26, 27].

Using this information, it is possible to evaluate the changes that a sample will undergo when subjected to different temperature conditions, making DSC an important technique to assess the temperature stability of a substance or mixture of substances and how they behave and react to specific temperatures [28].

High-performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) consists of injecting a mixture of substances (referred to as matrix) containing one or more molecules of interest (referred to as analytes) through a sequence of filters or columns to enable the separation of initial sample's constituents [29, 30]. Usually the samples pass through a column with which the constituents interact differently, depending on their chemical properties. Substances that have higher affinity for the column are more retained by it than substances with less or no affinity, which enables the separation of the mixture over time. As constituents leave the column, an adequate detector quantifies them and the apparatus then enables the determination of the amount of that constituent present in the mixture.

Sometimes the analytes do not possess properties that enable their detection; in these cases it is common to previously react them with substances that are capable of detection, a process defined as derivatization [31]. Dansyl chloride is one such agent and is commonly used in the derivatization of AA and functions by reacting with the amine groups of the AA and establishing a strong bond with the molecule [32, 33].

Lysine encapsulation efficiency (%Lys)

When considering NP for substance delivery, one of the most important properties is the amount of that substance that can be carried by the NP. In this work, the selected molecule was Lys. %Lys is the percentage of added Lys that was encapsulated within the NP matrix and can be measured either by a direct or an indirect method. In the direct method, the amount of Lys that is within the NP is quantified and compared with the total amount of Lys added during production, whereas in the indirect method the Lys that was not associated to the NP is separated and quantified. By determining the Lys that was not encapsulated, it is them simple to calculate the amount that was loaded into the NP.

Near-infrared spectroscopy

Near-infrared (NIR) spectroscopy consists of measuring the amount of radiation, in the NIR region of the spectra (750-2500 nm), that is absorbed by specific molecular bonds [34]. It functions by separating a beam of light into its component wavelengths and registering the amount of each of these wavelengths that is absorbed by a substance. Different molecular bonds interact differently with radiation of different wavelengths. By measuring the absorbance of each of these wavelengths, it is possible to determine the molecular bonds that are present in a sample and their relative abundance.

This is a highly flexible form of analysis that can be used to quickly determine a material's composition without altering the sample.

Simulated digestion processes

After ingestion, the nanosystems will be subjected to different conditions as they traverse the digestive tract and it is important to assess how they will behave in each one before considering an *in vivo* approach. Several different methods exist to assess the stability of NP, but the most commonly used rely either in collecting tissue or fluids from the animal and incubating the NP in them *ex vivo* [35] or the use of mimetic media and support apparatus to mimic the natural conditions [36]. The former is usually more similar to the real conditions and therefore is a more robust model, but it produces more variable results, due the wider diversity of natural conditions and animal variability and is sometimes more difficult to perform due to animal availability and tissue viability issues. The latter is normally regarded as a more simplistic model, differing more from the real conditions, but allows a greater understanding of the behavior in those specific conditions while still providing relevant information.

To determine if the NP were able to resist the several conditions of the bovine digestive tract, both methods were applied in this work, to mimic the digestion in the different organs, as well as to determine how they would react in the bloodstream. Both rumen inoculum and blood were collected from animals and

applied *ex vivo*, whereas the abomasum and intestinal conditions were mimicked using biomimetic media supplemented with adequate enzymes [37-40].

Cellular studies

In vitro cellular studies are powerful tools to study and determine interactions between NP and tissues and have tremendously reduced the need for *in vivo* studies over the years. These types of assays are primarily applied to determine cytotoxicity of molecules or particles and allow to assess if they would be safe for applications both in animal studies or actual products and applications.

Considering that the goal of this work was to develop a future feed application, the L929 mouse fibroblastic cell line was used, as they are support cells found all over the organism and are recommended for biomaterial safety evaluation for biomedical devices and food applications [41].

Cell viability assay

To study the viability of cells in the presence of NP the methylthiazolyldiphenyl-tetratozium bromide (MTT) assay was selected. This assay relies on the fact that viable and active cells are able to convert MTT into another compound, formazan, which has distinct colorimetric properties [42]. Formazan is quantified after incubation by spectrophotometry and correlated with the number of viable cells present. Cells incubated with the NP were then compared with cells without any treatment and the relative toxicity of the NP could be determined [43]. This study is very important to evaluate if the NP are safe for future *in vivo* studies and applications.

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"*Success is not final, failure is not fatal: it is the courage to continue that counts.*"

Winston Churchill

From mouth to mammary gland: a nanotechnology application in dairy cow nutrition for targeted lysine delivery

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